PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF THREE SELECTED ENSET (*Ensete ventricosum* (Welw.) Cheesman) VARIETIES; YANBULE, MESENA AND ENDALE THROUGH SHOOT TIP CULTURE

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Protocol Optimization for *In Vitro* Propagation of Three Selected Enset (*Ensete ventricosum* (Welw.) Cheesman) Varieties; Yanbule, Mesena and Endale through Shoot Tip Culture

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By

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APPROVAL SHEET

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As thesis research advisors, we hereby certify that we have read and evaluated this thesis prepared under our direction by Dejene Zinabu, entitled "Protocol Optimization for *in vitro* Propagation of Three Selected Enset (*Ensete ventricosum* (Welw.) Cheesman) Varieties; Yanbule, Mesena and Endale through Shoot tip culture". We recommend that it be submitted as fulfilling the thesis requirements.

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The author **Mr. Dejene Zinabu** was born in Southern Nations, Nationalities and Peoples` Region (SNNPR) of Ethiopia, at Guraghe Zone on May 23rd, 1989. He attended his elementary and junior school at Kulite and followed secondary and preparatory school at Wolkite. The author joined Arbaminch University in 2010 and obtained the Bachelor of Science Degree in Plant Science in June 2012. After graduation, he was employed at Wolkite University as a Graduate assistant from May 2013 up to joining Postgraduate Program at Haramaya University in October 2015 to pursue his study leading to M.Sc. degree in Biotechnology.

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ABBREVIATIONS AND ACRONYMS

2, 4-D	2, 4-Dichlorophenoxy Acetic Acid
AC	Activated Charcoal
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis Of Variance
BAP	6- Benzyl Amino Purine
CPRI	Central Potato Research Institute
CRD	Completely Randomized Design
CSA	Central Statistics Agency
DNA	Deoxyribonucleic Acid
EBW	Enset Bacterial Wilt
EIAR	Ethiopian Institute of Agricultural Research
IAA	Indole-3- Acetic Acid
IBA	Indole-3-Butyric Acid
IITA	International Institute of Tropical Agriculture
Kn	Kinetin
m.a.s.l	meter above sea level
MOA	Ministry of Agriculture
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
NABRC	National Agricultural Biotechnology Research Center
PCR	Polymerase Chain Reaction
PGR	Plant Growth Regulator
pH	Power of Hydrogen
RAPD	Random Amplified Polymorphic DNA
RH	Relative Humidity

TABLE OF CONTENTS

APPROVAL SHEET **ii**

STATEMENT OF THE AUTHOR **iii**

BIOGRAPHICAL SKETCH OF THE AUTHOR **iv**

ACKNOWLEDGEMENTS **v**

ABBREVIATIONS AND ACRONYMS **vi**

LIST OF TABLES **ix**

LIST OF TABLES IN THE APPENDIX **x**

LIST OF FIGURES IN THE APPENDIX **xi**

ABSTRACT **xii**

1. INTRODUCTION **1**

2. LITERATURE REVIEW **5**

2.1. Crop Description 5

2.1.1. Origin, taxonomy and distribution of enset 5

2.1.2. Morphological description 5

2.1.3. Ecology, agronomy and production status 6

2.2. Conventional Methods of Enset Propagation 6

2.3. Economic and Ecological Importance of Enset 7

2.4. Problem Associated With Enset Production 8

2.5. Plant Tissue Culture and Its Applications 8

2.5.1. Importance of *in vitro* propagation through shoot tip culture 9

2.5.2 The Role of Auxins and Cytokinins on Plant Tissue Culture 10

2.6. Enset biotechnological Approaches 12

2.7. Factors Affecting *in vitro* Propagation 13

3. MATERIALS AND METHODS **15**

3.1. The Experimental Site 15

3.2. Source of Experimental Material 15

3.3. Stock Solutions and Media preparation 16

3.3.1. MS media and growth regulators stock solutions preparation 16

3.3. 2. Medium Preparation 16

3.4. Shoots Culture Establishment Procedure 17

3.4.1. Sterilization of Explants 17

TABLE OF CONTENT CONTINUED

3.4.2. Shoot initiation 17

3.4.3. Shoot multiplication 18

3.4.4. Root induction and development 18

3.4.5. Plantlets acclimatization 19

3.5. Data collection 19

3.6. Experimental Design and Data Analysis 20

4. RESULTS AND DISCUSSION 21

4.1. Sterilization of the Explants 21

4.2. Effect of Cytokinin alone and combination with auxin on *In Vitro* Shoot Initiation 23

4.3. Effect of BAP or Kinetin alone or in combination with NAA on shoot multiplication 27

4.4. Effect of different auxins alone and with cyitokinin on root formation 34

4.5. Effect of acclimatization media mixture on plantlet survival in greenhouse 38

5. SUMMERY, CONCLUSION AND RECOMMENDATIONS 40

5.1. Summary and Conclusion 40

5.2. Recommendations 42

6. REFERENCES 43

7. APPENDICES 54

LIST OF TABLES

Table **Page**

1. General information about selected Enset varieties 15
2. Effect of *NaOCl* concentration on explants sterilization 22
3. Response of shoot initiation time taken under different initiation treatments 25
4. Response of different shoot multiplication parameters on shoot multiplication treatments 30
5. Rooting experiment results obtained from different rooting treatments 36
6. Enset plantlets survival percentage on different acclimatization media mixture 38

LIST OF TABLES IN THE APPENDIX

Appendix Table	Page
1. Nutrient composition and concentration of full strength MS medium	54
2. Plant growth regulators hormone supplied in the media	55
3. ANOVA Table for the effect of *NaOCl* on clean and live explants	56
4. ANOVA Table for Shoot Initiation	56
5. ANOVA Table for *in vitro* Shoot multiplication	57
6. ANOVA Table of rooting experiment	57

LIST OF FIGURES IN THE APPENDIX

Appendix Figure **Page**

1. Mother plant decapitation, decortication and Suckers multiplication 58
2. Five months old explant source stock plant and well prepard explant before sterilization 58
3. Aseptics sterilization techniques and explant trimming 59
4. Inoculated shoot tip explant and Contamination free initiated culture explants at 2% *NaOC* 59
5. Contaminated shoot tip culture 60
6. Active initiated bud and shoot after a month shoot initiation 60
7. Multiplied shoots and Shoot length 61
8. Well rooted plantlets and root length 61
9. Acclimatized Plantlets in the greenhouse 61

Protocol Optimization for *In Vitro* Propagation of Three Selected Enset (*Ensete ventricosum*(Welw.) Cheesman) Varieties; Yanbule, Mesena and Endale through Shoot tip Culture

ABSTRACT

Enset (Ensete ventricosum (Welw.) Chessman) has socio-cultural, ethno-medicinal and economic use values. Nearly all enset genotypes are being threatened by bacterial wilt disease upon conventional propagation. Therefore, this research was designed to develop mass in vitro propagation protocol for three elite enset varieties from shoot tip explants. The experiment was laid out in CRD with three replications in factorial arrangement. Apart from ethanol (70%), sodium hypochlorite (NaOCl) in three concentration levels (1, 2 and 3%) was used for surface sterilization. For shoot initiation explants were aseptically cultured on MS basal medium supplemented with 1.5, 2.5, 3.5 and 4.5 mg/l of BAP alone or in combination with 0.5 and 1mg/l of NAA, and incubated in dark for 4 weeks at room temperature. After 9 weeks of incubation, the initiated shoots were transferred to MS basal media supplemented with 2, 3.5, 5 and 6.5 mg/l of BAP or 2, 3.5, 5, 6.5 mg/l Kn alone or in combination with 0.5 mg/l of NAA. MS media without plant growth regulators were used as control. The initiated cultures were then incubated for five weeks at 25 ± 2^0 C and 16 hours photoperiod of white fluorescent light of (20 μ mol/ m^2/s) intensity. For root induction, about 5 to 8 cm of well regenerated shoots were cultured on half strength MS medium supplemented with 1.5, 3, 4.5 mg/l NAA or 1.5, 3 and 4.5 mg/l of IBA alone or in combination with 0.25 mg/l of BAP. Half strength MS medium without hormone was used as control. The cultures were maintained in a growth room for a month at a temperature of 25 ± 2^0C and 16 hour photoperiod. Plantlets were planted on different mixes of red soil, sand and compost in different mix ratios. Results of sterilization experiment showed that 2% NaOCl was found to be effective in giving contamination free explants. Compared to the control, almost all levels of hormone treatments had positive impact on all parameters measured from shoot initiation and multiplication, and rooting experiments. However, of all hormonal treatments, only specific hormone types, concentrations and mixes resulted in better performance in shooting and rooting experiments($p<0.05$). Results of acclimatization experiment showed that red soil and sand mixed in 2:1 ratio resulted in better plantlet survival. The three tested enset varieties responded differently to the treatments. Overall, the results of this study could be followed for in vitro propagation of the three tested enset varieties.

Key words: *Plantlets, Plant growth regulators, shoot tip, Varieties*

1. INTRODUCTION

Enset (*Ensete ventricosum* (Welw.) Chessman) is a perennial, herbaceous, monocarpic and monocotyledonous crop in the family Musaceae (Westphal, 1975). The genus Ensete consists of 6 species (all are diploid, 2n = 2x = 18) (Baker and Simmonds, 1953). Of the six commonly recognized species of Enset, *E. superbum* and *E. glaucum* grow wild in Asia while *E. perrieriin* in Madagascar and *E. gilletii*, *E.homblei* and *E. ventricosum* grow in eastern Africa (Simmonds, 1962). *E. ventricosum* is the sole cultivated member in the genus Ensete, and is cultivated exclusively in smallholder farming systems in southern and south western Ethiopia (Simmons, 1956). Domesticated enset is cultivated at altitudes ranging from 1, 200 to 3,100 meters above sea level. However, it grows best at elevations between 2,000 and 2,750 meters above sea level (Quimio and Mesfin, 1996; Brandt *et al.*, 1997).

According to Admasu *et al.* (1997), three enset based farming systems have been identified based on the level of priority given to enset cultivation in different zones and regions in Ethiopia. Enset is the first important food source in Gurage, Kembata, Sidama, Gedio, Hadya, Jemjem and Arero zones. It is a second important crop as co-staple food in Wolaita, Gofa, Kafa zones and Yem special woreda. It is planted as the third most important food crop in Wollega, Illubabora and in some parts of Southern region.

According to CSA (2016), the total area covered by harvested enset in Ethiopia is estimate to be 56, 261.1 hectares, of them, the total area covered by harvested enset in Southern Nations Nationalities and Peoples Regional State (SNNPRS) and Oromyia region is 38,516 and 17,631.9 hectares, respectively.

Enset is important in the Ethiopian economy, it is little investigated and remains an undervalued (*i.e* do not recognized how valuable or important) commodity crop. More than 20 percent of Ethiopia's population that are concentrated in the highlands of southern Ethiopia depend on enset for human food, fiber, animal forage, construction materials and medicines (Genet and Walander, 2004).

Enset is an important food crop produced in Ethiopia with great role in food security especially for southern and south western parts of the country. The demand of the crop is increasing throughout the country. It is a drought tolerant staple food crop (Genet and Walander, 2004). Different parts of it and processed products of several cultivated enset landraces are used to fulfill socio-cultural, ethno-medicinal and economic use-values (Temesgen *et al*., 2014). Amongst its uses, enset produces a starchy, carbohydrate rich food for human consumption. The fiber from the leaf and pseudo stem is comparable to that of Musa textiles (Afza *et al*., 1996) and is used for construction and rope making. The corm and pseudo stem of some Enset clones are used as traditional medicine to repair broken bones, facilitate abortions or discharge of placentas after birth in both cattle and humans, and possess antimicrobial properties (Holscher and Schneider 1998). A starch that can be used for paper, textile and adhesive industries is produced from enset (ESTC, 2003).

The crop is now threatened by biotic factors such as bacterial wilt disease caused by *Xanthomonas campestris* pathovar (pv) *musacearum* and fungal diseases caused by *Mycosphaerella musicola* and *Sclerotium rolfsi* (Quimio and mesefine, 1996; Genene, 2014). Moreover, in some areas, enset fields were completely destroyed due to diseases and farmers were forced to replace the field with other crops (Archaido, 1991). It is reported that up to 80% of enset farms are currently infected by Enset Xanthomonas Wilt (EXW) and devastated there crop. There is evidence that EXW is reducing enset yield and quality. Loss of a single enset plant in a family would mean loss of one man’s feed. This problem directly affects the livelihood of more than 20 million enset growing farmers in the country (SARI, 2014).

In nature, enset propagates by means of seeds and do not produce vegetative suckers. However, these can be induced to undergo clonal propagation by hollowing out an old corm and packing the hole with soil and dung before flowering (Simmonds, 1962). This conventional method of enset propagation is time consuming and is at the expense of a mature plant. Planting material produced through conventional method of propagation is one of the disease transmission mechanisms and, moreover, produces less number of suckers. Large numbers of disease free suckers are needed to distribute elite enset varieties, to establish a new plantation so as to replace the harvested or disease devastated enset farm

or to expand enset farm. Enset germplasm is currently maintained in Ethiopia in a field gene bank at risk of diseases, pests and adverse environmental conditions (Bezuneh, 2010). These limitations prevent an efficient and rapid production of Enset to meet the current demand of the crop. Thus, *in vitro* propagation is recommended to assist the conventional method. Tissue culture techniques may enhance mass *in vitro* propagation, production of disease free planting material, germplasm conservation and genetic improvement of the crop. Plant tissue culture techniques involve the growing and multiplication of totipotent cells, tissues and organs of plants on defined solid or liquid media comprising nutrients under an aseptic and controlled environment (George, 1993).

Micro propagation through shoot tip culture offers many advantages over conventional propagation methods such as higher rates of plantlets multiplication, production of true to type plants, regeneration of clean and disease free planting material with short time and limited space without season restriction (Edwin *et al*., 2008).

Plant growth regulators are inevitable for *in vitro* regeneration of crops in any artificial medium. Cytokinin triggers to induce shoot regeneration (Cheng *et al*., 2010). Cytokinin helps in shoot proliferation and auxins helps in rooting of proliferated shoots. However, the requirement of cytokinin and auxins depends on the variety and culture conditions (Cronauer and Krikorian, 1984).Genetic factors are considered to be a major contributor to the *in vitro* response of cultured tissues. *In vitro* plant regeneration is dependent on many factors such as plant genotype, explants type, culture medium, plant growth regulators and culture environment (Khanna and Raina, 1998). Though genotype based *in vitro* propagation protocol development is very necessary and genotype based optimization of growth regulator hormones is crucial for satisfactory *in vitro* propagation. Moreover, further optimizing and commercially utilizing micro propagation techniques for mass multiplication is evident. The development of an optimal micro propagation protocol would have a major significance in the propagation of good agronomic traits of enset clones for its dissemination to growers (Zeweldu and Ludders, 1998).

However, only limited efforts have been made to *in vitro* micropropagation and improvement of enset by using modern biotechnological approaches (Almaz *et al*., 2000). To achieve such an improvement, tissue culture procedures, which assure successful and efficient micro propagation needs to be developed. Few researchers have reported their research on enset tissue culture. For example, Taye (1984) and Almaz *et al*. (2000)

investigated the use of zygotic embryos for initiating enset cultures. Afza *et al*. (1996) reported the regeneration of enset through somatic embryogenesis. Mathew and Philip (1996) developed an *in vitro* propagation procedure for *Ensete superbum*, a wild relative of enset. Genet and Welander (2004) reported about effect of meristem wounding on efficient micro propagation of *Ensete ventricosum*. *In vitro* regeneration of disease free enset planting material from diseased enset shoot tip was reported by Genene (2014). In all of these investigations, only partial success and a low rate of multiplication were obtained. Moreover, there have been no experiment conducted using shoot tip explants for mass multiplication of these selected enset varieties.

Variety Yanbule, Mesena and Endale were registered, certified and nationally released from Areka Agricultural Research Centre based on their early maturity ability and high *Qocho* production potential (MOARD, 2009; MOA, 2010). Developing *in vitro* propagation protocol for rapid and mass multiplication of this important agronomic trait containing varieties is necessary, because the conventional method of enset propagation is time consuming to get appropriate corm for sucker multiplication. It is also prone to disease transmission and produces few numbers of suckers per corm with in long period of time.

Therefore, this experiment was designed to develop optimal protocol for *in vitro* propagation of three varieties of *Ensete ventricosum* with the following specific objectives.

Specific Objectives were to:

- Determine the optimal concentration of Sodium hypochlorite for shoot tip explants sterilization
- Determine the optimal concentrations and combinations of Cytokinins and Auxin for shoot initiation and multiplication
- Determine the optimal concentrations and combinations of Auxins and Cytokinin for root induction and development
- Optimize acclimatization media mixture to assess survival rates of plantlets in the greenhouse

2. LITERATURE REVIEW

2.1. Crop Description

2.1.1. Origin, taxonomy and distribution of enset

Ethiopia is enset's center of origin and holds a large number of enset geremeplasm collections from several geographical regions (Vavilove, 1951; Mikias, 2014). There have been efforts to understand local production practices and improve the conservation and use of the genetic resources of enset in order to enhance the mostly under exploited potential of this crop. Enset (*Ensete ventricosum*) consists of 6 7 species (Simmonds, 1962; Pursglove, 1972). Taye (1984) has also stated that 7 up to 8 species are known in the genus that means; stile within ambiguity about number of species in the genus Ensete.

In the past *Ensete ventricosum* was cultivated only in the south and south-western parts of Ethiopia, but the recurrent droughts have led to the expansion of enset cultivation to other parts of the country (Brandt *et al*., 1997). A wide adaptation within the species to altitude, soil and climate has allowed widespread cultivation in western Bale, south-western Oromia including south and east Shewa, Jima, Illubabor and Welega (Shank, 1994).

2.1.2. Morphological description

Enset is evergreen plant and it is composed of corm, pseudo stem and leaf as well as bended inflorescence during its maturity. A mature enset plant has an average height of 4–8 meters, but it could reach as high as 10 meters depending on the cultivars used and site conditions. The average basal diameter of its dilated pseudo stem is 0.5-1.0 meter. The pseudo stem, which is composed of leaf sheath, has a length of 1.0 to 2.5 meters in fully matured plants. Leaves are large with oblong blades and often long free petiole. The inflorescence grows from the center of plant, and fruits are small with large black non edible seeds (Bizuayehu, 2002). The vegetative growth habit of enset is similar to banana (Musa spp.) plants, but enset is not grown for the fruits; these contain mostly large and very hard seeds (Karlsson *et al*., 2013).

2.1.3. Ecology, agronomy and production status

Enset performs well in areas where the natural environment is steppe (Negash *et al*., 2012) and naturally occurs in forests and in humid areas (Baker and Simmonds,1953) it is suitable to grow in a wide range of climates, thus in large geographical areas. Temperature plays a significant role in the growth rate of enset. Accordingly, at the altitudinal ranges of 1500-2300 meters (Woyna Dega areas) where mean annual temperature is 15-20°C, enset grows fast and reaches full maturity in 5 to 7 years. On the other hand, in the high altitudes of 2300-3100 meters (Dega areas), where mean temperature drops to 10-15°C, it takes 8-10 years and sometimes up to 16 years (Shank and Ertiro, 1996) to reach full maturity. Enset need different agronomic practices like hoeing, weeding, and repetitive transplanting. Enset thrives best in fertile, well drained soils of moderately acidic to alkaline nature (Admasu Tsegaye, 2002).

Enset cover about 65% of the total crop production in the southern region of Ethiopia and productivity is very high compared to other crops but varies depending on edaphic factors, altitude, cultural practices and varietal differences (Genet *et al*., 2004). According to CSA (2014) report the production status of enset in Oromia region the estimated number of Enset trees harvested, in the 2013/2014 agricultural year, estimated were 34,461,351.0. Thus, the total produce in the form of *Amicho, Qocho*, and *Bula* was 7,061, 725.87; 8,303,792.24 and 320,991.67 quintals respectively. For instance at Jima, Borena, West Arisi and south west shewa the estimated number of enset tree harvested was 3,440,9 66.00; 1,495,355.00; 17,294,385.00 and 1,929,028.00 respectively. At SNNP Region the estimated number of enset tree harvested was 96,023,384.00. From thus total harvested enset tree produce *Amicho*, *Qocho* and *Bula* were 24,079,056.78; 26,410,479.01 and 904,896.31 quintals respectively. From the region at Gurage, Hadiya, Kmbata Tembaro and Sedama the estimated number of enset tree harvested was 3,760,845.00; 2,830,620.00; 1,211,004.00 and 56,320,910.00 respectively.

2.2. Conventional Methods of Enset Propagation

Wild species of enset propagate from seed (Bezuneh, 1996). Enset growers rarely use seed propagation, as germination of intact seed. However, Poor enset seed germination is attributed to the physical properties of the testa and size and physiology of the embryo.

Vegetative propagation, using corms, is a common practice in enset cultivation. A whole corm is planted or it is longitudinally split into two or four parts through the apex and each part is planted separately. The largest number of suckers, 35 suckers per half corm, was obtained from a three-year-old Halla clone when the mother plant was left undisturbed for one year, after removal of the apical bud. A study of one landrace indicates that splitting gives more as compared to intact one, but it still gives smaller number of suckers (Mulugeta *et al*., 2002).

2.3. Economic and Ecological Importance of Enset

Enset is an important multi-purpose and drought-tolerant crop, used for food and In terms of edible dry weight and energy, enset gives higher yield than other crops cultivated in Ethiopia (Admasu and Struik, 2001), fiber (Tsehaye and Kebebew, 2006) and traditional medicine (Nyunja *et al*., 2009). The food is rich in starch, is a good source of calcium and iron (Atlabachew and Chandravanchi, 2008) and has overall nutritive values similar to potato (Mohammed *et al*., 2013).

A mixture of scraped leaf sheath and pulverized corms, after fermentation in a pit, results in production of *Qocho*. *Qocho* is the main product consumed after making a pancake-like food. *Bulla* is another important food product from enset produced from solidified liquid after dehydrating a fresh mixture of scraped leaf sheath and pulverized corms. *Bulla* is consumed mainly as porridge, in gruel and as crumbled forms. Corms of some clones are cooked and consumed similar to roots and tubers of other crops. Processed products such as *Qocho* and *bulla* are sold in small town markets and also transported to the cities (spring, 1996). Leaves, as a wrapping material, and fibre are additional sources of income. Fibre, a by-product of enset in food processing, is a valuable raw material for household usage. Local fibre factories use this as an import substitute because the quality of enset fibre is equal to that of abaca and better than sisal (Bezuneh, 1996). Enset fiber accounts for more than 30% of the Ethiopian fibber production and its strength is equivalent to the fiber of Abaca (Brandt *et al*., 1997).

Enset plantations prevent soil erosion and conserve soils, hence, contributing to the sustainability of the farming system. Enset contributes positively to the local environment by improving the nutrient balance and increasing the fraction of organic matter in soil

(Asnakech, 1997). Enset is suitable for agroforestry and it is part of farming systems with high biodiversity which is environmentally sustainable (Bizuayhu, 2008; Negash *et al*., 2012), which is suggested for land improvement.

2.4. Problem Associated With Enset Production

The nature of the vegetative propagation and the long perennial life cycle of Enset make the enset production too difficult (Bezuneh, 2010) Convincing evidence indicates that enset is one of the most genetically understudied food security crops.

Moreover, the sustainability of Enset agriculture is threatened by fungal, bacterial and viral diseases. Bacterial wilt of Enset which caused by *Xanthomonas campestris* PV. Musacearum (Xcm) is the most important disease affecting Enset yield and general production coverage of the crop and conventional method of Enset propagation is prone to disease transmission (Quimio and Mesefin, 1996; Genene, 2014). There are also other production constraints such as, lack of improved varieties; most of Ensets need long time to crop maturity; so breeding good agronomic trait of the crop by conventional breeding system is difficult due to long life cycle; lack of effective disease and insect pests control measures.

2.5. Plant Tissue Culture and Its Applications

Plant tissue culture can be defined as the regeneration of whole plants *in vitro* using the culture of plant cells or plant tissues in a synthetic culture medium under controlled aseptic conditions (Garg, 2011). It is clear from literatures that the general phenomenon of tissue culture is the ability to produce embryoids, organs, tissues *etc*. from different explants like leaves, stem, cotyledons, microsporocytes and shoot tips. *In vitro* propagation *via* organogenesis usually involves four stages including initiation of cultures, multiplication of shoots, rooting of shoots, and acclimatization of plants (Cardoza, 2008).

There are two areas in which plant tissue culture methodology is important in plant product ion and breeding (Short, 1990).The first comprises current technologies such as clone multiplication, pathogen elimination, embryo rescue, haploid production and genetic conservation. The second, concerns situations in which genetic modification of plants can be induced by mutagenesis, somaclonal techniques, somatic hybridization and recombinant

DNA technology. These techniques for genetic modification depend upon micro propagation for the regeneration and multiplication of new characteristics. For instance, to use recombinant DNA technology in plant breeding a whole plant must be regenerated from transformed cells (Cardoza, 2008).

Elite varieties can be clonally propagated (Engelmann, 2010), endangered plants can be conserved (Pasqual *et al*., 2014), virus-free plants can be propagated by merustem culture (Sim, 2006), germplasm can be conserved (Mathur, 2013), and secondary metabolites can be produced by cell culture (Vanisree *et al*., 2004; Mathur, 2013). Plant tissue culture now has direct commercial applications as well as value in basic research into cell biology, genetics and biochemistry (Garg, 2011).

Tissue culture provides excellent advantages over traditional propagation, including a high multiplication rate, physiological uniformity, the availability of disease-free material all the year round, rapid dissemination of new plant materials throughout the world, uniformity of shoots, and faster growth in the early growing stages compared to conventional materials (Arias, 1992). Regeneration from callus and pollen cultures helps to produce homozygous, pure-breeding lines of plants for hybrid production and genetic studies and also to improve the efficiency of *in vitro* selection (Lalremsiami and Robert, 2013).

2.5.1. Importance of *in vitro* propagation through shoot tip culture

The term *in vitro* propagation is also used to describe the *in vitro* techniques because cultures are started with very small pieces of plants and small shoots are thereafter propagated. The foundation of *in vitro* propagation is the so called totipotency theory, which states that cells are autonomic and, in principle, capable of regenerating to give a complete new plant (Pierik, 1993).

In vitro propagation is yet the most important innovative to overcome barriers in multiplication of elite plant species. The most important commercial technique for *in vitro* propagating plants is known as shoot tip culture. *In vitro* propagation of banana through shoot tip cultures is useful in the rapid multiplication of desirable disease free clones. New and effective means of propagating bananas would be advantageous over the conventional use of sucker material, for germplasm maintenance, exchange and transportation (RAO, 1994). Major applications of shoot tip culture are suitable not only for the large-scale

production of uniform and vigorously growing propagates for field establishment, but also for germplasm conservation. In the former already existing shoot tips are stimulated to multiply rapidly, while in the latter, the multiplication rate is slowed down (Vuylsteke, 1989). Moreover, shoot tip culture is important to regenerate disease free planting material (Genene, 2014).

2.5.2 The Role of Auxins and Cytokinins on Plant Tissue Culture

Hormones are organic compounds naturally synthesized in higher plant, which affect the growth and development of plants by exerting a profound influence on physiological processes (Hopkins and Huner, 2009). Apart from natural hormones, synthetic hormones have been developed which act-like the natural ones. These synthetically produced hormones are commonly called plant growth regulators and have biological activity which equals or exceeds that of the equivalent endogenous hormones (Gaspar *et al.*, 1996). In tissue culture, plant growth regulators are important media components in determining the development and developmental pathway of the plant cells. Growth regulators are used in different proportions to break dormancy and enhance shoot formation as the apical dormancy is under control of these growth regulators (Madhulatha *et al.*, 2004).

The requirement for growth substance varies depending on the type and source of the explants and on their endogenous level (Bhojwani and Razdan, 1996).The cytokinins and auxins are of importance in *in vitro* culture as the later are concerned with root formation, the former is mainly required in the media for shoot formation and growth of buds (North *et al.*, 2012). These growth regulators are required in combination in the media as it is always the manipulation and variation of auxins and cytokinins levels that can successfully change the growth behavior of plant cultures (Gonzales, 1994).

The irreversible degradation of cytokinins, catalyzed by cytokinin oxidase, is an important mechanism by which plants modulate their cytokinin levels (Brugiere *et al.*, 2003). Cytokinins like BAP, furfurylamino purine (kinetin), thidiazuron (TDZ) and zeatin have been commonly employed alone and/or in combination with other cytokinins or/and auxins for proliferation and multiplication of different plant species (Gaspar *et al.*, 1996). Of these, BAP and Kinetin are the most active, cheapest and easily autoclaved without exhaust. BAP is known to reduce the apical meristem dominance and induce both auxiliary and adventitious shoot formation from meristematic explants in banana (Khalid, 2011).

However, the application of higher cytokinins concentrations inhibits elongation of adventitious meristems and the conversion into complete plants.

The effectiveness of BAP over other cytokinins in inducing multiplication of shoot tip cultures has been reported in different cultivars of bananas. BAP has a marked effect in stimulating the growth of axillary and adventitious buds and foliar development of shoot tip cultures (Buah *et al.*, 2010). 6-Benzyl amino purine (BAP) is the most commonly preferred cytokinin (Vuylsteke, 1989). Genotype of given plant species determines its buds proliferation *in vitro*. Apart from the influence of genotypes, shoot proliferation rate and elongation are influenced by cytokinin types and their concentration. Adenine based cytokinins are used in several Musa spp. for *in vitro* propagation (Gubbuk and Pekmezci, 2004).

The shoots obtained require rooting. This process is stimulated by endogenous auxin and by exogenous application of this hormone, which checked by expression of the Ranilla luciferase (RUC) gene in root generation part (Tyburski and Tretyn, 2004). Auxins induce cell division, cell elongation, apical dominance, adventitious root formation, and somatic embryogenesis (Rai, 2007). Auxins like NAA, 2, 4 dichlorophenoxyacetic acid (2, 4-D), indole-3 acetic acid (IAA), IBA *etc* are the most frequently used in plant tissue culture alone and in combination. IBA has advantage over some other synthetic auxins. IBA can be used with MS media for root formation of Amritasagar and Sabri banana cultivars through shoot tip culture (Ferdous *et al*., 2015).

The type of morphogenesis that occurs in a plant tissue culture largely depends on the ratio and concentration of auxins and cytokinins in the medium. With this regard, adventitious and auxiliary shoot proliferation occurs when the ration of auxin to cytokinin is low, whereas root induction of plantlets, embryogenesis and callus initiation occur when the ratio of auxin to cytokinin is high (George *et al*., 2008).

Combinations of BAP with auxins such as indole acetic acid (IAA) were also used for *in vitro* shoot multiplication of bananas. Although BAP alone stimulates shoot proliferation in bananas (Resmi and Nair, 2007). 4.0 mg/L BAP with 1.0 mg/L IAA in liquid medium was best for shoot multiplication and shoot height during micro propagation of Banana ‘Basrai’ (Aish *et al*., 2007). Adventitious shoots were also regenerated from compact calli that were

cultured on MS medium containing 5 mg/l BA and 0.1 mg/l NAA in banana *in vitro* propagation (Amornwat and Kamnoon , 2007).

2.6. Enset biotechnological Approaches

Due to very low attention to enset research, very little or no benefits have yet been gained from application of biotechnological approaches. Although the anticipated role biotechnology can play in enset development is enormous, this has not been exploited to improve its production or productivity.

Analysis of genetic diversity among cultivated *Ensete ventricosum* populations from Essera and Kefficho, southwestern part of Ethiopia using inter simple sequence repeats (ISSRs) marker reported by (Dagmawit and Endashaw, 2011). Similarly, Almaz *et al.* (2002) has been used Amplified Fragment Length Polymorphism (AFLP) to assess intra specific genetic diversity of enset landraces. Moreover, According to Temesgen *et al.* (2015) report SSR markers specific to *E.ventricosum* were developed through pyrosequencing of an enriched genomic library.

In addition, genetic transformation of enset have been on progress in Ethiopia at National Agricultural Biotechnology Research Center Holetta, and Kenya at Nirobi, in a collaborative work to regenerate bacterial wilt resistance plantlets of enset in tissue culture (Endale Gebre, personal communications).

Enset *in vitro* propagation

Research on tissue culture of enset was fairly recent. Apart from micro propagation, extensive researches had focused on: *Ensete ventricosum in vitro* regeneration from zygotic embryos of stored seeds (Mulugeta and Van Staden, 2003); corm and leaf tissue explants culture (Almaz *et al.* 2000) and was reported to be 2-3 enset shoots per explant per four weeks subculture. Enset shoots were regenerated from callus (Afza *et al.,* 1996; Morpurgo *et al.,* 1996).

In other studies, disease free enset plantlets were regenerated from bacterial diseased enset through shoot tip culture (Genene, 2014). Similarly, effect of enset shoot tip explants wounding to induce multiple shoots was also reported and explants apical wounding increased shoot multiplication than unwounded explants (Genet and Welander, 2004).

Comparative *in vitro* response performance of Banana and Enset was also done by (Zeweldu, 1997). Mulugeta and van Staden (2004) have reported that 3 to 4 leaves/shoot for *in vitro* source Enset explants and 2 to 3 leaves/shoot for shoots regenerated from greenhouse source explants.

2.7. Factors Affecting *in vitro* Propagation

Microbes compete adversely with plant tissue cultures for nutrients and their presence often results in increased culture mortality or can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Oyebanji *et al.*, 2009). The problem of microbial contamination can be combated through effective surface sterilization of explants for *in vitro* culture initiation. Basically, sterilization is the process of making explants contamination free before establishment of cultures.

Various sterilization agents are used to decontaminate plant tissues for *in vitro* cultures. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explants to various sterilants, the sequences of using these sterilants has to be standardized to minimize explants injury and achieve better survival (CPRI, 1992). In essence, requirements on the type concentration and time of exposure differ from one plant to another and for different parts of plants depending on their morphological characters like softness /hardness of the tissue (Srivastava *et al.*, 2010). In addition, the sterilizing solutions containing the explants are continuously stirred during the sterilization period.

One of the most common problems associated with the *in vitro* establishment of many monocotyledonous and woody species is the deleterious effects of oxidized phenols (Chakrabarty *et al.*, 2007). Many plants produce dark phenolic substances after wounding. Accumulation of such compounds in medium adversely affects the growth and survival of *in vitro* explants. The phenols are synthesized by the plants and in many cases excreted and then oxidized (Ozyigit, 2008). In tissue culture studies, phenolic substances, especially oxidized phenols generally affect *in vitro* culture negatively (Arnaldos *et al.*, 2001).

The rate of shoot proliferation is also affected by the degree of browning of the shoot tip tissues which phenomenon is attributed in part to tissue death resulting from oxidative stress incurred at the cut surfaces of tissues or to oxidation of plant phenolic compounds in

presence of light (Leng *et al.*, 2009). Micro propagation could be highly useful for enset but has unfortunately proved to be exceptionally difficult due to phenol exudation (Zeweldu and Ludders, 1998).The problem of phenol oxidation was minimized by adding activated charcoal to the medium in enset culture (Genet and Welander, 2004).

Role of Activated Charcoal for *In vitro* Propagation

Phenolics are inhibitory to cellular growth. Quinine substance which are produced as a result of oxidation of phenolic compound and will gradually enter the tissues cultured on the medium and further represses the activities of other enzymes and as a result, poison other contents of the medium (Feng *et al.*, 2007). The major problem during the micro propagation is lethal browning caused by the exudation of phenolic compounds from excised portion of explants (Ishtiaq *et al.*, 2013). Explant browning caused by oxidation of phenolic compounds resulting from injuries the isolation of explants. It leads to the death of explant and failure of shoot regeneration (North *et al.*, 2012).

The phenolic oxidation or brown exudates accumulation can be significantly reduced by activated Charcoal (Thomas, 2008). Activated charcoal (AC) has a very fine network of pores, an extraordinarily large surfaces area and volume that gives it a unique adsorption capacity (Baker *et al.*, 1992). AC is often used in tissue culture to improve cell growth and development. The main benefit is its adsorption of inhibitory substances in culture media. Addition of AC in the medium as well as increased sub culturing frequency reduced this problem in rice culture considerably (Soniya and Sujitha, 2006). Nguyen *et al.* (2007) tried to eradicate phenolic problem in sorghum by using AC in the medium. AC (1-5 g/l) had a positive effect on reducing the black pigments released into the media by immature embryos.75-100% embryos of wild enset (W01) genotype become black *in vitro* in the absence of Activated Charcoal .These rate of blackening were reduced to less than 25% when 5g/l AC was added to the media (Mulugeta and van staden, 2004).

3. MATERIALS AND METHODS

3.1. The Experimental Site

The experiments were conducted in National Agricultural Biotechnology Research Center (NABRC) at Holetta, which is located at 34 Km to the west of Addis Ababa. Geographically; it is found at Altitude, Latitude, and Longitude of 2391 m.a.s.l., 9° 3` N, and $38^{o}$ 30` E, respectively.Mean annual maximum and minimum temperatures are 21°C and 6°C, respectively (EIAR, 2013).

3.2. Source of Experimental Material

Three enset varieties, namely Yanbule, Mesena and Endale were obtained from Areka Agricultural Research Center and were grown at Holetta NABRC green house.

Table 1 General information about selected Enset varieties

Agronomic Characteristics	**Varieties**		
	Yanbule	**Mesena**	**Endale**
Adaptation area	Mid to high altitude	Mid to high altitude	Mid to high altitude
Altitude	1200-3100	1200-3100	1200-3100
Seed rate (Suckers/ha)	2222	2222	2222
Planting date	March or June on rain fed Agriculture	March or June on rain fed Agriculture	March or June on rain fed Agriculture
Days to crop maturity	3 to 4 years	4 to 5 years	3.5 to 4.5 Years
Un-Squeezed Qocho yield (ton/ha/year)	33.8	25.27	20.8
Squeezed Qocho yield (ton/ha/year)	22.8	21.81	14.4
Year of released	2009	2010	2009
Breeder/Maintainer	Areka ARC/SARI	Areka ARC/SARI	Areka ARC/SARI

Source- MOARD (2009) and MOA (2010)

Two years old mother plant pseudostem was cut down and cross cuts/ incisions were made on the growing meristem so as to stimulate the production of lateral buds. The pseudostem of the mother plants was cut transversely 4cm above the collar region and then the apical meristem is removed leaving a cavity of 3cm diameter and 4 cm depth (Appendix Figure

1A). This was done to overcome the apical dominance. These corms were surface sterilized by dipping in 0.3 % Bavistine for 45 min, and allowed to dry for three days and then planted in a sterilized soil at Holetta NABRC greenhouse. Decapitation and decortications of mother plants was made, which activated the lateral buds giving rise to more side shoots.

3.3. Stock Solutions and Media preparation

3.3.1. MS media and growth regulators stock solutions preparation

In this study, Murashige and Skoog (1962) MS media was used along with the proper type and concentration of plant growth regulators. A stock solution for each of the MS components (Appendix Table 1) were weighed in recommended amounts and completely dissolved in double distilled water by grouping it in six group based on their solubility nature (Appendix Table 1) in order to reduce bottles consumption and stored at +4°C. The stock solutions of MS nutrients, vitamins and amino acid were prepared fresh every month. Iron and Na_2.EDTA mix stock solution was protected from light by storing the solution in bottles wrapped with aluminium foil.

The plant growth regulators(Appendix Table 2) were prepared in a 1mg: 1ml ratio after being weighed and dissolved using drops of 1 N NaOH or ethanol, allowed to be completely dissolved and the volume was adjusted by adding double distilled water and stirred on magnetic stirrer. The stock solutions were stored at +4 ^{0}C for immediate/few days use.

3.3. 2. Medium Preparation

After preparing MS and plant growth regulators (PGRs) stock solutions and appropriate mixing, the pH of the medium was adjusted at 5.8 and autoclaved at a temperature of 121° C with a pressure of 103 Kpa for 20 minutes (Murashige and Skoog, 1962). An Antibiotic (Cefataxime) solution was sterilized by using Millipore filter paper with pore size of 0.22 micrometer diameter to avoid denaturation during autoclaving and it was added for shoot initiation and sterilization trial media only. After autoclaving, the culture medium was stored in a clean dust free chamber for 4-5 days before use in order to check for any media contamination.

3.4. Shoots Culture Establishment Procedure

3.4.1. Sterilization of Explants

After five months of enset suckers growth, suckers of explants source were carefully removed, without damaging the rhizome portion and brought to the preparation room of the Laboratory. The roots and outer tissues of the suckers were removed with the help of a sharp knife. Sheaths were removed until the shoot measured to about 3.0 cm in length and 3.0 cm width at the base (Appendix Figure 2B).

The prepared explants were washed using tap water and rinsed with absolute ethanol for 3 min. Finally the explants were washed thoroughly under running tap water with Largo and Tween 20 with slight shaking and washed tile removed Largo and Tween 20.

The explants were then dipped in 70% (v/v) ethanol for 10 min and then rinsed three times with sterile distilled water in aseptic condition in the laminar air flow hood (Appendix Figure 3A). The explants were then first treated with 1, 2 or 3% sodium hypochlorite (in the form of Chlorox bleach) with 3 drops of Tween 20 for 20 minutes for disinfection. Thereafter, the outer surface of the explant that was exposed to sterilizing agent was removed and the explants were trimmed at both ends by using sterilized surgical blade and forceps (Appendix Figure 3B). Disinfection was again done in the same way using *NaOCl* for 10 minutes without Tween 20. Explants were then rinsed with sterile distilled water 3 times and trimmed to have size of about 0.8- 1cm length and 0.5 - 1 cm diameter with its subtending corm. Then after shoot tip explants inoculated on cefataxime containing MS medium to collect explants contamination data under different concentration *of NaOCl.*

3.4.2. Shoot initiation

Well sterilized explants by 2% *NaOCl* (other explants sterilization material and procedure was used as explants sterilization experiment) were longitudinally sliced into two halves dissecting the meristem into two where each half was placed onto the shoot initiation medium in culture jars with the corm tissue end half way embedded into the media. Shoot initiation medium was MS basal medium enriched with 30 g/l Sucrose, 1 g/l Active Charcoal, 4.5 g/l agar, and 500 mg/l filter sterilized cefataxime and supplemented with 1.5, 2.5, 3.5 and 4.5 mg/l of BAP alone or in combination with 0.5 and 1mg/l of NAA (Adane,

2013). The jars were sealed with cling film and incubated at room temperature and preserved at dark growth room without any illumination for two successive weeks. Then after, culture transferred to light condition at 25 ± 2^0 C and 16 hours photoperiod of white fluorescent light of (20 µ mol/ m^2/s) intensity. The experiment conducted with three replications and each treatment was contained 15 shoot tip explants. After 4 weeks of incubation, the initiated shoot tip explants were then transferred to the same fresh initiation media and maintained for three weeks by sub-culturing in order to get multiple initiated shoot and to enhance shoot multiplication rate for shoot multiplication experiment.

3.4.3. Shoot multiplication

The initiated shoots were then cultured for two weeks on PGRs free MS medium before transferring to shoot multiplication media to prevent carryover effect of initiation hormone. The initiated shoot explants were then transferred to shoot multiplication media that consisted of MS basal media including vitamins and amino acid with 30 g/l sucrose, 4.5 g/l of agar, 1g/l activated charcoal and 25 mg/l Ascorbic acid supplemented with 2, 3.5, 5 and 6.5 mg/l of BAP or 2, 3.5, 5, 6.5 mg/l Kn alone or in combination with 0.5 mg/l of NAA (Aish *et al.*, 2007). MS media without exogenous plant growth regulators were used as control both in initiation and multiplication experiments. The initiated cultures were incubated on multiplication media for five weeks at 25 ± 2^0 C and 16 hours photoperiod of white fluorescent light of (20 µ mol/ m^2/s) intensity and randomly arranged in the lab. All multiplication treatments contained 15 uniform and equal size initiated shoots with little corm at the base with three replications.

3.4.4. Root induction and development

The multiplied shoots were then cultured for two weeks on PGRs free MS medium before transferring to root induction and development medium to prevent carryover effect of shoot multiplication hormones. For root induction, about 5 to 8 cm of well regenerated shoots were cultured on half strength MS medium consisting of 30 g/l sucrose, 1 g/l Activated Charcoal, 5 g/l agar, and supplemented with 1.5, 3, 4.5 mg/l NAA or 1.5, 3 and 4.5 mg/l of IBA alone or in combination with 0.25 mg/l of BAP in 250ml culture jars (Mathew and Philipe, 1996; Bizuayhu, 2002). Half strength MS medium without hormone was used as a control. The cultures were maintained in a growth room for a month at a temperature of 25 ± 2^0C and 16 hour photoperiod provided with white florescent tube. Each treatment

contained 15 explants within three replication and treatments were randomly arranged in the lab.

3.4.5. Plantlets acclimatization

Well regenerated plantlets with shoots, roots and leaves were gently removed from the culture jars and the roots were washed in tap water to remove traces of agar. The plantlets were then socked for 24 hr in water on water bath finally transplanted into acclimatization cell try which contained different acclimatization media mixture: red soil, compost and sand mixture at 2:1:1 ratio, red soil in combination with compost mixture at 2:1 ratio or red soil and sand only mixture at 2:1 ratio. Each acclimatization cell try with plantlet were covered with white plastic and red cheese cloth to maintain humidity and kept in the green house at 25^0 C ± 2^0 C, 16hrs photoperiod with 2700 lux light intensity. From all varieties 15 plantlets were transplanted on to each acclimatization medium mixture. After two weeks the plastic and red cheese cloth cover was removed and plantlets survival rate was assessed after four weeks.

3.5. Data collection

The following *in vitro* plant growth parameters were collected from the five subsequent experiments

Percentage of explants that were contamination free and survived: number of explants which was free from contamination and survived counted from each replication and converted into percentage.

Days to shoot initiation: days needed by each explant to initiate shoot was recorded from the first day of culturing and an average day was computed for each replication.

Number of shoots per explant: total number of shoots produced from initiated shoot explant after five weeks of inoculations was counted and mean was computed for each replication.

Shoot Length: length of each multiplied shoot was measured in centimetres from base to the tip using ruler (cm) after five weeks of culturing.

Number of Leaf: The average number of leaves per each *in vitro* multiplied shoot

Shoot fresh and dry weight: fresh and dry weight of each multiplied shoots after five weeks culturing measured by electronic sensitive balance for each replication.

Days of root emergency: days needed by each explant to emerge root were recorded from the first day of culturing and an average day was computed for each replication.

Number of roots per shoot: the total number of induced roots was counted for each cultured shoot and mean was computed for each replication.

Length of root: length of induced roots was measured in centimetres, from base to tip for each root.

Root induction Percentage: the number of rooted shoot was counted after four weeks of culturing and converted into percentage for each replication

Plantlets survival rate on acclimatization: total number of acclimatized plantlets was counted and converted into percentage after four weeks green house hardening.

3.6. Experimental Design and Data Analysis

The experimental design for this study was completely randomized design (CRD) as factorial arrangement by three replications. Data recorded were subjected to ANOVA and significant differences among treatments were determined by Duncan's multiple range tests at 5% level of significance by using SAS software for the differentiation of the effect of growth hormones, varieties and growth hormones by varieties interaction.

4. RESULTS AND DISCUSSION

4.1. Sterilization of the Explants

To solve *in vitro* contamination problem, surface sterilization of explants before culture was done with *NaOCl* in the form of Chlorox bleach at different concentration while other conditions were maintained aseptic. Sodium hypochlorite is the most commonly used disinfectant for surface sterilization of banana explants (Cronauer and Krikorian 1984; Mendes *et al*., 1996; Muhammad *et al*., 2004). Compared to the control where *NaOCl* was used for sterilization of explants, explant contamination was significantly reduced and survival rate improved (Table 2). Analysis of variance (ANOVA) also revealed that concentration of *NaOCl* and its interaction with varieties had significant difference ($P < 0.05$) in overcoming contamination and improving survival of explants (Table 2 and Appendix Table 3).

Most explants in the control group (*NaOCl* free) were contaminated after 10th day, giving a contamination rate for the control group of 100% by day 23 for all varieties (Table 2 and Appendix Figure 5A). Although 1% *NaOCl* resulted in greater percentage of contamination free survived explants, values were significantly lower than that of 2 and 3% *NaOCl* in all varieties (Table 2 and Appendix Figure 5B). In this study, the potential for *NaOCl* to result in high percentage of contamination free survived explants was in an order of 2>3>1%, suggesting 2% *NaOCl* is optimal concentration for effective sterilization of the three tested varieties of Enset. Although 3% *NaOCl* prevented explant contamination, some explants were found to be dead; suggesting increasing concentration of *NaOCl* beyond 2% has negative impact too for these tested explants.

Table 2. Effect of *NaOCl* concentration on explants sterilization

Varieties	Concentration of *NaOCl* (% v/v)	Explants immersion time(min) in *NaOCl* in double sterilization	No of explants/ replication	Mean No of Live and Clean explants/ treatment	Percentage of Live and Clean explants (%)(mean±SD)
Mesena	0	20 and 10	4	0	0.0 ±0.0[g]
	1	20 and 10	4	2	50.0 ±25.0[de]
	2	20 and 10	4	4	100 ±0.0[a]
	3	20 and 10	4	3.33	83.3 ±14.4[bc]
Yambule	0	20 and 10	4	0	0.0 ±0.0[g]
	1	20 and 10	4	1.33	33.3 ±14.4[f]
	2	20 and 10	4	4	100 ±0.0[a]
	3	20 and 10	4	3.67	91.7 ±8.3[ab]
Endale	0	20 and 10	4	0	0.0 ±0.0[g]
	1	20 and 10	4	2.33	58.3 ±14.4[d]
	2	20 and 10	4	4	100 ±0.0[a]
	3	20 and 10	4	3	75.0 ±0.0[c]
CV					18.24
ISL					*

CV= Coefficient of Variation, SD= Standard Deviation, ISL= Interaction Significance Levels, *= Significant, Data represent the mean of four replicates. Means followed by same letter in the column do not differ statistically at $p=0.05$.

These results are in agreement with that of Dayarani *et al*. (2013) who found 100% clean surviving ornamental banana by using *NaOCl* as disinfectant. Similarly, Reza *et al*. (2013) also reported high percent (99%) clean explants obtained when commercial bleach (chlorox) was used on some *Musa* species. Shahnawaz *et al*. (2014), however, reported that sterilization of banana explants to get clean surviving explants was poor (55 to 100%) with sodium hypochlorite alone. The variation of current study from former previous result may be attributed to explants source. Similar previous findings showed that explants contamination rate depends on several plant and environmental related factors such as species, age, explant source or size and prevailing weather condition (Rout *et al*., 2000). Additionally, Oyebanji *et al*. (2009) showed that Sodium hypochlorite produced the highest reduction in bacterial and fungal contamination at time intervals between 20-45 minutes. Generally, the results of the present study showed that sterilization with low concentration of *NaOCl* had poor response; likewise an increase in concentration of *NaOCl* had adverse effect on the culture.

4.2. Effect of Cytokinin alone and combination with auxin on *In Vitro* Shoot Initiation

Shoot tip culture was initiated using BAP and NAA treatments in different concentrations alone or in combination. Analysis of Variance showed that hormones, varieties and their interaction showed significant effect on days to shoot initiation ($p < 0.05$) and a well-defined trend was observed (Table 3 and Appendix Table 4).

Compared to the control, both BAP in sole and in combination with NAA showed significant positive effect on shoot initiation days of the three varieties of enset at all concentration levels. Variety Yanbule required minimum days for shoot initiation as compare to other varieties which observed at 2.5 mg/l BAP with 0.5 mg/l NAA, but it had no significant difference as compared to 1.5 mg/l levels of BAP (Appendix Figure 4). Minimum days for shoot initiation for variety Mesena and Endal achieved at MS media fortified with 2.5 mg/l BAP with 0.5mg/l and 2.5 mg/l BAP alone, respectively.

In the present study shoot initiation was much earlier as compared to results reported by Mulugeta and Staden (2004) experiment. They reported MS media gelled with 11 g/l of agar and supplemented with 2.5 mg/l BAP was best for other Enset genotype early shoot initiation in which single Enset shoot initiation was observed after two weeks. The most probable reasons for this early culture initiation in the present experiment may be due to genotype difference and the low amount of solidifying agent (4.5 g/l agar) used that can increase nutrient and PGR hormone uptake of explants.

Shoot initiation days of three varieties were significantly higher at the highest used concentration of BAP alone or in combination with NAA. Consistent with the present study, results of Muhammad *et al.* (2013) showed that banana culture took 15-21 days for shoot initiation on media supplemented with high concentration of BAP (5.0 mg/l) in combination with 1.0 mg/l IAA. Similarly, According to Ferdous *et al.* (2015) single shoot was developed at 5.0 mg/l BAP (12.0 days on Amritasagar and 14.0 days on Sabri banana varieties). Sazdur *et al.* (2013) also noticed optimum concentration of phytohormones is required for early shoot induction of banana. Moreover, the present results showed that addition of NAA on BAP enriched media significantly facilitate shoot initiation on both Yanbule and Mesena varieties. Similarly, previous workers have supported the use of cytokinin with auxin combination for *in vitro* initiation of banana (Arinaitwe *et al.*, 2000).

In general, Mesena and Endale initiated lately as compared to Yanbule variety. This late shoot initiation may be due to genotype difference.

Table 3. Response of shoot initiation time taken under different initiation treatments

Varieties	Hormones(mg/l) BAP	NAA	Days required for shoot initiation
Endale	0	0	14.8 ± 0.75^{a}
	1.5	-	9.82 ± 1.02^{efghijkl}
	2.5	**-**	$\mathbf{9.0 \pm 1.0^{mn}}$
	3.5	-	10.32 ± 2.12^{defghij}
	4.5	-	11.5 ± 1.4^{cde}
	1.5	0.5	10.8 ± 1.71^{cdefgh}
	2.5	0.5	9.98 ± 0.9^{efghijkl}
	3.5	0.5	9.95 ± 1.19^{efghijkl}
	4.5	0.5	10.63 ± 0.0^{cdefghi}
	1.5	1	11.93 ± 2.59^{cd}
	2.5	1	11.05 ± 0.0^{cdefg}
	3.5	1	10.86 ± 0.74^{cdefgh}
	4.5	1	11.09 ± 0.08^{cdefg}
Mesena	**0**	**0**	13.35 ± 1.18^{ab}
	1.5	-	9.76 ± 0.79^{fghijk}
	2.5	-	9.79 ± 0.0^{efghijk}
	3.5	-	10.51 ± 1.3^{cdefghi}
	4.5	-	10.85 ± 1.19^{cdefgh}
	1.5	0.5	10.14 ± 1.56^{efghijk}
	2.5	**0.5**	$\mathbf{8.39 \pm 0.65^{mno}}$
	3.5	0.5	9.39 ± 1.54^{ghijk}
	4.5	0.5	9.15 ± 0.81^{hijk}
	1.5	1	11.38 ± 1.02^{cdef}
	2.5	1	11.25 ± 0.79^{cdef}
	3.5	1	9.78 ± 0.0^{efghijk}
	4.5	1	9.8 ± 1.23^{efghijk}
Yanbule	0	0	12.16 ± 0.82^{c}
	1.5	**-**	$\mathbf{7.44 \pm 0.23^{no}}$

Table 3. Cont…

Varieties	Hormones (mg/l)		Days required for shoot initiation(Mean±SD)
Yanbule	BAP	NAA	
	2.5	-	7.95 ± 0.62^{mn}
	3.5	-	9.31 ± 0.47^{hijklm}
	4.5	-	9.26 ± 1.4^{hijklm}
	1.5	0.5	7.86 ± 0.98^{mn}
	2.5	**0.5**	$\mathbf{7.0 \pm 0.5^{o}}$
	3.5	0.5	8.56 ± 0.53^{klmn}
	4.5	0.5	9.0 ± 0.5^{ijklmn}
	1.5	1	9.23 ± 0.99^{hijklm}
	2.5	1	8.32 ± 1.1^{lmn}
	3.5	1	8.92 ± 0.94^{ijklmn}
	4.5	1	$10.74 \pm 0.12^{efghijkl}$
CV			10.7
Interaction Significant levels			*

CV= Coefficient of Variation, SD= Standard Deviation, Data represent the mean of three replicates with fifteen explants for each treatment. Means followed by same letter in the column do not differ statistically at p=0.05.

4.3. Effect of BAP or Kinetin alone or in combination with NAA on shoot multiplication

Analysis of Variance showed that hormones, varieties and their interactions had highly significant ($p< 0.0001$) effect on number of shoot, length of shoot, shoot fresh and dry weight and results are shown in Table 4 and appendix Table 5.

As indicated in Table 4, number of shoot per explant of all varieties was significantly higher under BAP and Kn alone or in combination with NAA at all concentration levels when compared to control. Significantly best medium for shoot multiplication was obtained on 5 mg/l BAP for variety Yanbule (Table 4 and Appendix Figure 7B). However, with BAP concentration increment from 5 mg/l to 6.5 mg/l number of shoot per explant was significantly reduced. In consistent with present study, Khalafalla *et al*. (2007) concluded that shoot number decreases as BAP concentration increases above some optimum level. For variety Mesena, media supplemented with 6.5 mg/l BAP was better than other treatments (Table 4 and Appendix Figure 7A). For variety Endale, high shoot number was recorded at 6.5 mg/l Kn alone. But it had no significant difference as compared to 5 mg/l BAP. There was a great deal of enhancement of multiple shoot formation when high concentration of Kn was used alone. However, combination of Kn with NAA hormones produced less shoots for this variety.

From the three tested varieties Endale was responded poorly in terms of number of shoot under media supplemented with different hormone concentrations and combination, but Yanbule was the best performing variety in terms of producing maximum number of shoots. This shows that *in vitro* enset shoot multiplication is affected by genotype difference. The higher concentration of BAP produced malformed/stunted shoot on varieties Yanbule and Endale. Similarly, according to Buising *et al*. (1994) the application of higher concentrations of BAP (7 mg/l) on banana culture inhibits elongation of adventitious meristems and their conversion into complete plants.

In an interaction of Kn with NAA showed the same trend with that of Kn alone in terms of shoot number in all tested varieties. This suggests that addition of NAA to Kn did not enhance shoot multiplication. However, Adane (2013) observed maximum number of shoot per explant (3.08) on Cytokinin with auxin combination at 5 mg/l BAP + 0.5 mg/l

NAA on banana shoot multiplication. The difference between the current result and that of Adane (2013) may be due to the difference in the synergetic nature of cytokinin used.

The number of enset shoot per explant obtained from the present study was found to be better than that reported (2 to 3) by Almaz *et al.,* (2000). Similarly, Mulugeta and van Staden (2004) obtained 3.7 shoots and 3.5 shoot buds per single shoot after splitting the enset explants in two and culturing them separately, which is less than what observed in this study. This difference may be attributed to genotype difference and explants corm size.

Shoot length per explant of all tested varieties was significantly higher under BAP and Kn in sole or in combination with NAA at all concentration levels when compared with the control. In both cytokinins (BAP and Kn) the shoot length is inversely related to their respective concentration (Table 4).The maximum shoot length was recorded in MS medium supplemented with 2 mg/l BAP for variety Yanbule (Table 4 and Appendix Figure 7C). However, further increase in BAP to 6.5 mg/1significantly reduced the shoot length (Table 4). In the case of variety Mesena the longest shoot was recorded at 3.5 mg/l BAP. Increasing BAP concentration from 0 to 3.5 mg/l showed an increase in shoot length. However, further increase in BAP to 6.5 mg/1, significantly reduced the shoot length. But it had no significant difference as compared to 5 mg/l BAP and 2 mg/l Kn supplemented medium for this variety.

Whereas in variety Endale the longest shoot length was obtained on MS medium fortified with 3.5 mg/l Kn and increase in kinetin from 0 to 3.5 mg/l showed a significant increase in shoot length. However, shoot length decreased afterward. Similarly, according to Woeste *et al*. (1999) the reduction in shoot length at higher concentration of BAP and Kn might be due to the toxic effects of ethylene produced at higher cytokinin concentration. Result of the present study indicated that the higher concentration of' BAP (up to 3.5 mg/1) was good for maximum shoot length, but beyond that level, shoot length will decrease.

In line with this study, Mulugeta and van Staden (2004) had conducted experiment on the effect of growth regulators and activated charcoal on plantlet regeneration from enset shoot tip explants and reported 3-5 cm shoot length on media with 2.5 mg/l BAP and 7gm/l AC, which was less than shoot length reported in the present experiment. The higher shoot length response observed in the present study may be due to the addition of low concentration (1 gm/l) of activated charcoal on shoot multiplication media. High

concentration of activated charcoal adsorbs not only phenol compounds, but also growth hormones and some organic and inorganic substances that may retard growth of shoots.

Number of leaves per shoot of variety Endale and Mesena was significantly higher under PGR supplemented medium at all concentration levels when compared with the control. Whereas in case of variety Yanbule, control medium had no significant difference when compared to the high concentration of Kn (6.5 mg/l) and it was not also significantly varied from the three levels of Kn (2, 3.5 and 5mg/l) combined with 0.5 mg/l NAA. The present result showed that up on decreasing the concentration of cytokinins, the number of shoots will decrease, but the leaf number and shoot length will increase (Table 4).

Though 3.5 mg/l of BAP gave higher number of leaves per shoot for variety Yanbule and the leaves appeared malformed, when hormone elevated afterward. On the other hand, 6.5 mg/l Kn found to be best in terms of yielding maximum leaves per shoot on variety Endale. However, this treatment did not significantly vary as compared to other treatments such as 3.5 mg/l BAP as well as 3.5 mg/l Kn in sole and (5 and 6.5 mg/l Kn) in combination with 0.5 mg/l NAA. In the case of variety Mesena highest leaf number per shoot recorded at 2 mg/l and 5 mg/l BAP. But it was not statistically significant from 3.5 mg/l of BAP and 2, 3.5 mg/l Kn alone and 5 mg/l Kn to 0.5 mg/l NAA combination.

In line with the present study, Al-Amin *et al*. (2009) conducted *in vitro* propagation experiment on banana and found that the maximum number of leaves (7 leaves/shoot) was produced on the medium supplemented with 7.5 mg/l BAP with 0.50 mg/l NAA. Similarly, Mulugeta and Staden (2004) have reported that 3 to 4 leaf per shoot for *in vitro* source explants and 2 to 3 leaves for shoots regenerated from greenhouse source explants on enset.

Table 4. Response of different shoot multiplication parameters on shoot multiplication treatments

Varieties	Hormones(mg/l)			Different shoot multiplication parameters				
	BAP	Kn	NAA	NSH(Mean± SD)	LSH(Mean ± SD)	NLF(Mean ± SD)	SHFW(Mean ± SD)	SHDW(Mean ±SD)
Endale	**0**	**0**	**0**	**0.33 ± 0.36**^{q}	**1.33 ± 0.15**^{r}	**1.20 ± 0.2**^{q}	**0.02 ± 0.00**^{w}	**0.008 ± 0.0008**^{p}
	2	-	-	1.44 ± 0.14^{p}	1.7 ± 0.22^{pq}	1.51 ± 0.25^{op}	0.13 ± 0.01^{v}	0.015 ± 0.0012^{o}
	3.5	-	-	1.93 ± 0.12^{lmnop}	2.37 ± 0.31^{nop}	**2.20 ± 0.2**^{ijklm}	0.67 ± 0.01^{nopqr}	0.042 ± 0.0^{jkl}
	5	-	-	3.33 ± 0.12^{fgh}	2.05 ± 0.41^{opq}	1.73 ± 0.12^{nop}	0.38 ± 0.02^{stuv}	0.024 ± 0.001^{no}
	6.5	-	-	1.60 ± 0.2^{nop}	1.55 ± 0.39^{q}	1.53 ± 0.12^{op}	0.15 ± 0.00^{v}	0.01 ± 0.0^{p}
	-	2	-	1.87 ± 0.23^{lmnop}	1.77 ± 0.05^{pq}	1.73 ± 0.12^{nop}	0.35 ± 0.02^{stuv}	0.017 ± 0.0^{o}
	-	**3.5**	-	1.87 ± 0.23^{lmnop}	**3.97 ± 0.27**^{hi}	**2.07 ± 0.12**^{klmn}	**1.42 ± 0.01**^{fgh}	**0.076 ± 0.0002**^{ef}
	-	5	-	1.67 ± 0.31^{nop}	2.15 ± 0.13^{opq}	1.93 ± 0.12^{lmno}	0.56 ± 0.0^{qrst}	0.028 ±0.0002^{lmno}
	-	**6.5**	-	**3.47 ± 0.12**^{fg}	1.63 ± 0.25^{pq}	**2.27 ± 0.23**^{hijklm}	0.24 ± 0.01^{v}	0.013 ± 0.0^{o}
	-	2	0.5	1.53 ± 0.11^{np}	1.69 ± 0.02^{pq}	1.47 ± 0.12^{op}	0.31 ± 0.02^{tuv}	0.013 ± 0.0^{0}
	-	3.5	0.5	1.9 ± 0.12^{lmnop}	2.61 ± 0.50^{mno}	1.73 ± 0.06^{nop}	0.63 ± 0.06^{opqrs}	0.04 ± 0.004^{jklm}
	-	5	0.5	2.27 ± 0.11^{ijklmn}	3.12 ± 0.05^{klm}	1.93 ± 0.11^{lmno}	0.85 ± 0.1^{lmnopq}	0.054 ± 0.0004^{ghij}
	-	6.5	0.5	2.87 ± 0.12^{hij}	1.74 ± 0.18^{pq}	1.87 ± 0.12^{lmnop}	0.33 ± 0.04^{tuv}	0.012 ± 0.0^{o}
Mesena	0	0	0	0.66 ± 0.24^{q}	2.85 ± 0.3^{lmn}	1.40 ± 0.2^{q}	0.26 ± 0.05^{uvw}	0.0145 ± 0.0^{p}
	2	-	-	2.67 ± 0.12^{hijk}	4.72 ± 0.02^{g}	**2.53 ± 0.12**^{fghi}	1.21 ± 0.2^{hijk}	0.067 ± 0.0^{fgh}
	3.5	**-**	**-**	3.07 ± 0.12^{fghi}	**5.26 ± 0.49**^{def}	**2.47 ± 0.58**^{ghij}	**1.48 ± 0.05**^{ef}	**0.088 ± 0.002**^{cde}

Table 4. Cont…

Varieties	Hormones(mg/l)			Different shoot multiplication parameters				
Mesena	BAP	Kn	NAA	NSH(Mean ± SD)	LSH(Mean ± SD)	NLF(Mean ± SD)	SHFW(Mean± SD)	SHDW(Mean± SD)
	5	-	-	3.27 ± 0.06^{fgh}	4.92 ± 0.41^{fg}	**2.53 ± 0.5^{fghi}**	1.25 ± 0.03^{hij}	0.067 ± 0.0^{fgh}
	6.5	**-**	**-**	**7.47 ± 0.42^{b}**	3.52 ± 0.51^{ijk}	2.13 ± 0.12^{jklmn}	0.59 ± 0.17^{pqrst}	0.033 ± 0.001^{klmn}
	-	2	-	2.0 ± 0.0^{klmnop}	5.0 ± 0.32^{efg}	2.27 ± 0.12^{hijklm}	1.31 ± 0.01^{ghi}	**0.079 ± 0.001^{de}**
	-	3.5	-	2.0 ± 0.0^{klmnop}	4.7 ± 0.45^{g}	2.33 ± 0.31^{hijkl}	1.11 ± 0.01^{ijkl}	0.068 ± 0.028^{fg}
	-	5	-	2.53 ± 0.3^{ijkl}	3.85 ± 0.51^{ijk}	1.8 ± 0.35^{mnop}	0.84 ± 0.03^{lmnopq}	0.052 ± 0.0^{hij}
	-	6.5	-	3.27 ± 0.23^{fgh}	3.56 ± 0.36^{ijk}	2.07 ± 0.12^{klmn}	0.69 ± 0.08^{nopqr}	0.044 ± 0.0^{ijk}
	-	2	0.5	2.0 ± 0.2^{klmnop}	3.91 ± 0.45^{hij}	2.07 ± 0.12^{klmn}	0.95 ± 0.19^{klmn}	0.048 ± 0.0^{ijk}
	-	3.5	0.5	2.27 ± 0.12^{ijklmn}	3.63 ± 0.48^{ijk}	1.8 ± 0.2^{mnop}	0.82 ± 0.00^{mnopqr}	0.049 ± 0.001^{ij}
	-	5	0.5	2.47 ± 0.42^{ijklm}	4.60 ± 0.05^{gh}	2.33 ± 0.23^{hijkl}	1.01 ± 0.1^{jklm}	0.059 ± 0.0^{ghi}
	-	6.5	0.5	4.53 ± 0.5^{cd}	3.21 ± 0.24^{jkl}	2.07 ± 0.12^{klmn}	0.53 ± 0.05^{rstu}	0.026 ± 0.0^{mno}
Yanbule	0	0	0	0.66 ± 0.24^{q}	3.38 ± 0.18^{ijkl}	2.60 ± 0.3^{ghij}	0.87 ± 0.06^{lmnop}	0.045 ± 0.0^{ij}
	2	-	-	2.53 ± 0.23^{ijkl}	**9.09 ± 1.14^{a}**	3.27 ± 0.5^{bc}	**2.55 ± 0.12^{ab}**	**0.11 ± 0.002^{a}**
	3.5	-	-	3.53 ± 0.9^{fg}	5.69 ± 0.58^{cde}	**3.73 ± 0.12^{a}**	**2.71 ± 0.12^{a}**	0.093 ± 0.043^{bcd}
	5	**-**	**-**	**9.13 ± 0.42^{a}**	5.60 ± 0.03^{cdef}	3.47 ± 0.64^{b}	1.65 ± 0.1^{cdef}	0.09 ± 0.0^{bcde}
	6.5	-	-	4.27 ± 0.58^{de}	3.92 ± 0.7^{hij}	3.0 ± 0.2^{bcdef}	0.9 ± 0.02^{lmno}	0.092 ± 0.0^{bcde}
	-	2	-	1.80 ± 0.2^{mnop}	6.40 ± 0.45^{b}	3.27 ± 0.12^{bc}	1.95 ± 0.18^{b}	**0.11 ± 0.008^{a}**

Table 4. Cont…

Varieties Yanbule	Hormones(mg/l)			Different shoot multiplication parameters				
	BAP	Kn	NAA	NSH(Mean ±SD)	LSH(Mean ± SD)	NLF(Mean ± SD)	SHFW(Mean± SD)	SHDW(Mean± SD)
	-	3.5	-	2.07 ± 0.31[klmnop]	4.99 ±0.14[efg]	3.07 ± 0.12[bcde]	1.43 ± 0.39[efgh]	0.08 ± 0.0[def]
	-	5	-	3.07 ± 0.42[fgh]	5.15 ± 0.19[efg]	3.13 ± 0.58[bcd]	1.48 ± 0.49[efgh]	0.089 ± 0.004[bcde]
	-	6.5	-	5.0 ± 0.8[c]	4.84 ± 0.2[g]	2.6 ± 0.0[ghij]	1.34 ± 0.12[ghi]	0.077 ± 0.001[def]
	-	2	0.5	2.53 ± 0.46[ijkl]	5.11 ± 0.22[efg]	2.67 ± 0.12[defghi]	1.55 ± 0.0[defg]	0.081 ± 0.002[def]
	-	3.5	0.5	1.93 ± 0.23[lmnop]	5.96 ± 0.83[bcd]	2.73 ± 0.31[defgh]	1.90 ± 0.32[bc]	0.101 ± 0.001[b]
	-	5	0.5	2.67 ± 0.42[hijk]	6.09 ± 0.09[bc]	2.93 ± 0.12[cdefg]	1.79 ± 0.22[bcd]	0.1 ± 0.006[bc]
	-	6.5	0.5	3.6 ± 1.27[ef]	5.65 ± 0.4[cdef]	3.0 ± 0.56[bcdef]	1.53 ± 0.26[efgh]	**0.11 ± 0.0[a]**
CV				**12.9**	**10.2**	**11.6**	**14.4**	**15**
ISL				***	***	**	***	***

NSH= Number of shoot per explants, LSH= Length of shoot (cm), NLF=Number of leaves per shoot, SHFW= Shoot fresh weight per shoot (gm), SHDW=Shoot dry weight per shoot (gm), CV= Coefficient of Variation, SD=Standard Deviation, ISL= Interaction Significant Levels, ** = Significant and *** = highly significant. In a column, means followed by the same letter are not significantly different at the 5% level by DMRT.

Compared to control, shoot fresh weight per shoot of all tested varieties was significantly higher under BAP and Kn alone or in combination with NAA at all concentration levels. However, in case of Yanbule variety high concentration of BAP (6.5 mg/l) had no significance difference as compared to control. In contrast with this study, Previous findings on banana shoots multiplication, maximum shoot fresh weight was obtained on high concentration of BAP (6mg/l) with IAA (0.35mg/l) combination (Munguatosha *et al.*, 2013). This result variation was happened may be due to response difference on synergetic effect of BAP with IAA or genotype response variation.

The effect of BAP at 3.5 mg/l was significantly higher than other tested treatments on shoot fresh weight per shoot of Yanbule and also it was best for variety Mesena. But it had no significance difference compared to 2 mg/l BAP for variety Yanbule. As indicated in Table 4. Shoot fresh weight of all varieties showed well-defined trend among hormone treatments. For instance, when concentration of BAP increased to 3.5 mg/l, Shoot fresh weight also significantly increased; however, further increase in BAP to 6.5 mg/1 significantly reduced shoot fresh weight of variety Yanbule. In case of variety Endale Significantly higher shoot fresh weight per shoot was observed on 3.5 mg/l Kn.

Remarkable variations were observed between shoot dry weight treatments. Among all treatments, significantly higher shoot dry weight per shoot was recorded at 2 mg/l BAP on variety Yanbule. But it had no significant difference as compared to 2 mg/l Kn alone and 6.5 mg/l Kn in combination with 0.5 mg/l NAA. Whereas the highest shoot dry weight per shoot of Mesena recorded was at 3.5 mg/l BAP. However, it had no significance difference as compared to 2 mg/l Kn. When BAP concentration was increased from control to 3.5 mg/l, shoot dry weight per shoot appeared to increase, but decreased afterwards, this might be due to the inhibitory effect of cytokinins at higher concentrations. Kn also showed related trend as compared to BAP on this variety. In case of Endale variety, significantly higher shoot dry weight per shoot was observed at media fortified with 3.5 mg/l Kn.

4.4. Effect of different auxins alone and with cyitokinin on root formation

Two auxins namely IBA and NAA alone as well as IBA in combination with BAP were supplied in half strength MS medium to check their effect on different rooting parameters and results are shown in Table 5. Analysis of Variance showed that varieties and hormones interaction had highly significant ($p < 0.0001$) effect on Days of Root induction, number of root per shoot, Length of Root per shoot and root induction percentage (Table 5 and Appendix Table 6).

Compared to the control, NAA and IBA alone or in combination with BAP showed significant positive effect on root formation of all tested varieties of enset. Number of roots per shoot, number of days to root emergence, length of root/shoot and root induction percentage significantly varied between treatments in three varieties. For Yanbule variety the highest roots number per shoot, root length per shoot and root induction percentage recorded at combination of 3 mg/l IBA with 0.25 mg/l BAP (Table 5 and Appendix Figure 8A). However, it had no significant difference as compared to 1.5 mg/l NAA for root length of Yanbule. Day's required for root emergency was lowest at 1.5 mg/l IBA combined with 0.25mg/l BAP, but found to increase with increasing IBA concentration afterwards for Yanbule variety (Table 5). In line with the present study, Almaz *et al.* (2000) reported that root formation of enset occurs in less than two weeks of transferring the shoot to a medium supplemented with 1 mg/l IBA, 0.2 mg/l IAA and 0.2 mg/l BAP in combination. The present result showed that root was initiated earlier (6.96 days) as compared to the previous finding on banana (Rahman *et al.,* 2005) at 1 mg/l IBA. This early root initiation in the present study is probably due to genotype response difference and hormone combination preference.

Among the three tested varieties, significantly higher root induction percentage was observed in variety Yanbule. It was best at hormone combination of IBA with BAP as compare to IBA and NAA alone. This study result showed that addition of small concentration of BAP on elevated concentration of IBA enabled better rooting response. Similarly, Mathew and Philipe (1996) obtained very good rooting response at media supplemented with 3m/l IBA in combination with 0.5 mg/l BAP on *Ensete superbum.*

In case of Endale variety, root induction percentages were significantly higher at 1.5 mg/l NAA; however, it was not significant as compared to 4.5 mg/l IBA. While, maximum root length per shoot was recorded at 1.5 mg/l IBA in combination with 0.25 mg/l BAP; But it was not statistically significant as compared to 1.5 mg/l NAA. Number of days to root emergence was also found to be shorter at 1.5 mg/l NAA than other treatments. However, number of roots per shoot was significantly higher at NAA (4.5 mg/l) for this variety. In contrast to this study, concentration of IBA at 2.0 mg/l exhibited superiority in terms number of roots per shoot (6.1) on banana rooting (Munguatosha *et al.*,2013) and additionally Rahman *et al*. (2005) also noticed maximum banana root number per shoot at IBA. Similarly, according to Al-amin *et al*. (2009) findings on banana rooting experiment, 6.0 roots per shoot obtained at IBA 1.0 mg/l + 0.5 mg/l IAA. This result variation might be occurred due to genotype difference.

Whereas in Mesena variety significantly higher number of roots per shoot, root induction percentage and minimum days for root emergency was recorded at 3 mg/l NAA (Table 5 and Appendix Figure 8B). But, it had no significant difference as compared to 4.5 mg/l NAA and 3 mg/l IBA in combination with 0.25 mg/l BAP on root induction percentage. Length of root/shoot was significantly higher at low concentration of NAA (1.5 mg/l) than other treatments. But, it had no significant difference as compared to 1.5 mg/l IBA. In harmony to the present study, the maximum root length per shoot (5.96cm) was obtained from enset shoots cultured on media with lowest concentration (0.5mg/l) of IBA (Genene, 2014). Similarly, Molla *et al.* (2004) got 2.60-5.67 cm range of root length in low concentration of IBA 0.5 mg/l on banana root induction.

In general, NAA was found to be best for root formation of Endale and Mesena varieties than IBA alone or combination with BAP. Among the three varieties, Yanbule gave better results in terms of the above mentioned parameters. This is suggesting that varietal difference evokes varying response to the same PGRs.

Table 5. Rooting experiment results obtained from different rooting treatments

Varieties	Hormones(mg/l)			Date of Root emergency (Mean ±SD)	Number of Root/shoot (Mean±SD)	Length of Root(cm)/ shoot(Mean ±SD)	Root Induction Percentage (%)(Mean ±SD)
	IBA	NAA	BAP				
Endale	0	0	0	**13.46 ± 1.06^{bc}**	1.67 ± 0.29^{opqr}	1.88 ± 0.47^{p}	40 ± 0.0^{def}
	1.5	-	-	9.95 ± 0.86^{hij}	2.67 ± 0.29^{cd}	3.53 ± 0.58^{klm}	33.33 ± 11.5^{ef}
	3	-	-	10.4 ± 0.41^{ghi}	2.44 ± 0.1^{cdefg}	1.66 ± 0.56^{p}	46.67 ± 11.5^{de}
	4.5	-	-	12.4 ± 1.26^{cde}	2.44 ± 0.17^{cdefg}	2.89 ± 0.58^{no}	**60.0 ± 0.0^{bc}**
	-	1.5	-	**9.0 ± 0.0^{lm}**	1.67 ± 0.29^{opqr}	**5.51 ± 0.85^{f}**	**60 ± 0.0^{bc}**
	-	3	-	9.81 ± 0.64^{hij}	2.0 ± 0.0^{ghijklm}	3.84 ±0.45^{jklm}	46.67 ± 11.5^{de}
	-	4.5	-	10.9 ± 0.61^{fgh}	**3.5 ± 0.0^{a}**	3.06 ±0.53^{mno}	46.67 ± 11.5^{de}
	1.5	-	0.25	12.8 ± 1.24^{cd}	1.67 ± 0.28^{opqr}	**5.68 ± 0.66^{ef}**	26.67 ± 11.5^{g}
	3	-	0.25	9.68 ± 0.59^{ijk}	2.17 ± 0.28^{fghijk}	5.07 ± 0.55^{ghi}	33.33 ± 11.5^{ef}
	4.5	-	0.25	10.1 ± 0.36^{hij}	2.17 ± 0.28^{fghijk}	5.03 ± 0.59^{ghi}	33.33 ± 11.5^{ef}
Mesena	0	0	0	14.38 ± 0.5^{ab}	1.83 ± 0.28^{ijklmn}	2.23 ± 0.28^{op}	40 ± 0.0^{def}
	1.5	-	-	11.96 ± 0.7^{def}	2.0 ± 0.0^{ghijklm}	**5.83 ± 0.53^{e}**	40 ± 0.0^{def}
	3	-	-	10.6 ± 0.68^{ghi}	1.39 ± 0.29^{pqr}	4.31 ± 0.56^{ijkl}	33.33 ± 11.5^{ef}
	4.5	-	-	11.49 ±0.55^{efg}	1.20 ± 0.39^{s}	4.29 ±0.48^{ijkl}	46.67 ± 11.5^{cde}
	-	1.5	-	10.9 ± 0.07^{fgh}	2.67 ± 0.29^{cd}	**5.99 ± 0.38^{e}**	26.67 ± 11.5^{g}
	-	**3**	-	**8.79 ± 0.44^{lm}**	**3.17 ± 0.29^{ab}**	4.28 ± 0.45^{ijkl}	**60 ± 0.0^{bc}**
	-	4.5	-	9.85 ± 0.19^{hijk}	2.58 ± 0.36^{cdef}	3.89 ±0.14^{jklm}	**60 ± 0.0^{bc}**
	1.5	-	0.25	12.64 ± 0.99^{cd}	1.44 ± 0.09^{opqr}	4.47 ± 0.58^{hijk}	46.67 ± 11.5^{de}
	3	-	0.25	11 ± 0.51^{fgh}	2.28 ± 0.25^{defghi}	4.99 ± 0.48^{fghi}	**60 ± 0.0^{bc}**
	4.5	-	0.25	11 ± 0.0^{fgh}	1.83 ± 0.29^{ijklmn}	5.1 ± 0.87^{fghi}	33.33 ±11.5^{ef}
	0	0	0	**15.33 ± 0.7^{a}**	1.17 ± 0.29^{s}	3.36 ±0.27^{klmn}	40 ± 0.0^{def}
Yanbule	1.5	-	-	9.63 ± 0.3^{ijkl}	2.33 ± 0.14^{cdefgh}	8.06 ± 0.62^{bc}	66.67 ± 11.55^{b}
	3	-	-	11.33 ±0.47^{efg}	2.8 ± 0.19^{b}	5.43 ± 0.44^{fgh}	66.67 ± 11.55^{b}
	4.5	-	-	13.07 ± 0.48^{cd}	1.5 ± 0.17^{opqr}	4.59 ± 0.06^{ghij}	66.67 ± 11.55^{b}
	-	1.5	-	8.91 ±0.39^{jklm}	2.22 ± 0.38^{defghi}	**9.89 ± 0.8^{a}**	66.67 ± 11.55^{b}
	-	3	-	8.54 ± 0.72^{lm}	2.77 ± 0.19^{bc}	7.49 ± 0.38^{cd}	53.33 ±11.55^{bcd}

Table 5. Cont…

Varieties	Hormones(mg/l)			Days of Root Induction(Mean ±SD)	Number of Root /shoot(Mean ±SD)	Length of Root(cm)/shoot(Mean ±SD)	Root Induction Percentage(%) (Mean ±SD)
Yanbule	IBA	NAA	BAP				
	-	4.5	-	9.45 ± 0.38^{ijkl}	2.39 ± 0.35^{cdefg}	5.51 ± 0.2^{efg}	60 ± 0.0^{bc}
	1.5	-	0.25	**6.96 ± 0.22^{n}**	1.67 ± 0.33^{lmnopqr}	8.32 ± 0.42^{bc}	53.33 ±11.55^{bcd}
	3	-	0.25	7.86 ± 0.8^{m}	**3.36 ± 0.38^{a}**	**9.67 ± 0.48^{a}**	**73.33 ± 11.55^{a}**
	4.5	-	0.25	9.54 ± 0.31^{ijkl}	2.1 ± 0.0^{ghijklm}	6.79 ± 0.61^{d}	60 ± 0.0^{bc}
CV				**5.9**	**12**	**10.5**	**19**
ISL				***	***	***	***

CV= Coefficient of Variation, ISL= Interaction Significant Level, ***= highly significant and SD= Standard Deviation. In a column, means followed by the same letter are not significantly different at the 5% level by DMRT. Each treatment had three replications with five well regenerated shoot per replication.

4.5. Effect of acclimatization media mixture on plantlet survival in greenhouse

In the present study, during hardening stage some plantlets were found wilting in the first week of transferring and some leaves were dried up. This may be due to unrestricted loss of water from their leaves or low hydraulic conductivity of roots and root-stem connections (Kumar and Rao, 2012). However, after 21 days of hardening new leaves were developed from each enset shoot. Gradually the plantlets started growing and the leaf number increased as the plant height increased. After a period of four weeks plantlets survival percentage data was taken (Table 6).

Table 6. Enset plantlets survival percentage on different acclimatization media mixture

Acclimatization media mixture	Varieties	No of transplanted plantlets to acclimatization media	No of survived plantlets during acclimatization	Plantlets survival percentage (%)
Red soil, Sand and Compost(2:1:1 ratio)	Yanbule	15	14	93.3
	Mesena	15	10	66.7
	Endale	15	12	80
Red soil and compos t(2:1 ratio)	Yanbule	15	12	80
	Mesena	15	9	60
	Endale	15	8	53.3
Red soil and Sand(2:1 ratio)	Yanbule	15	15	100
	Mesena	15	15	100
	Endale	15	15	100

Among all acclimatization media mixtures, red soil in combination with sand at 2:1 ratio was found as the best acclimatization media mixture for all tested varieties in which 100% plantlets could survive (Table 6 and Appendix figure 9A). The next high survival percentage (93.3, 80.0 and 66.7) was obtained for local control treatment (red soil, compost and sand mixture at 2:1:1 ratio) for variety Yanbule, Endale and Mesena, respectively. Whereas, plantlets survival percentage (80.0, 60.0 and 53.3) using red soil with compost only was less than the control treatment on Yanbule, Mesena and Endale, respectively.

In agreement with the present study, the use of sufficiently porous substratum that allows adequate drainage and aeration has been recommended for fast and efficient acclimatization of *in vitro* regenerated plants (Dunstan and Turner, 1984). Also the present research results highly in consistent with previous research finding that sand enhances porosity and drainage. Similarly, Anbazhagan *et al.* (2014) obtained around 87% of banana plants after four weeks under greenhouse, which transplanted on pots containing sand, soil and vermin compost in the ratio of 1:1:1. Generally, the present acclimatization experiment was highly efficient. Hence, enset plantlet acclimatization with red soil to sand at 2:1 ratio is best for higher plantlet survival percentage.

5. SUMMERY, CONCLUSION AND RECOMMENDATIONS

5.1. Summary and Conclusion

Enset (*Ensete ventricosum* (Welw.) Chessman) is a perennial, herbaceous, monocarpic and monocotyledonous crop in the family Musaceae. It is a drought tolerant staple and food security crop. Different parts of it and processed products of enset are used to fulfill socio-cultural, ethno-medicinal and economic use-values.

Conventional propagation methods of enset are a clonal propagation through a corm which makes planting of enset require large amount of materials and space. It is also takes long period of time to get the mother corm and to produce mature plants. This conventional clonal propagation system is prone to disease transmission. The crop is threatened by biotic factors such as bacterial wilt and fungal diseases. Moreover, in some areas, enset fields are completely destroyed due to severe disease problems where often farmers are forced to replace the field with other crops. Time to enset maturity takes long years which make enset culture a challenge to its growers. Usually large numbers of disease free suckers are needed to establish a new plantation for either replacing harvested crop or disease devastated enset farm or to expand enset production. Thus, *in vitro* propagation is recommended to assist the conventional method. The plant tissue culture methods provide a novel way for the asexual multiplication of enset plants free from diseases and in unlimited number.

Therefore, in the present study *in vitro* mass propagation protocol were optimized for nationally certified and released very early maturing enset varieties to boost good quality disease particularly pathogen free plantlets production. The experiment was laid out in CRD with three replications in factorial arrangement and data was analyzed by SAS software.

In the shoot initiation experiment shoot tip explants were cultured on MS media contained 30g/l of sucrose, 4.5g/l agar, 1g/l activated charcoal, 500 mg/l cefataxime and supplemented with 1.5, 2.5, 3.5 and 4.5 mg/l BAP alone and in combination with 0.5 and 1 mg/l NAA was used as initiation treatments. Also full strength MS media without cefataxime enriched with 2, 3.5, 5 and 6.5 mg/l BAP and Kn alone and in combination with 0.5 mg/l NAA were used for shoot multiplication experiments. Whereas, half strength

MS media supplemented with 1.5, 3.5 and 4.5 mg/l NAA and IBA alone and in combination with 0.25 mg/l BAP was used for rooting experiment. For all experiments MS media devoid of hormone used as control. Different enset plantlet acclimatization media mixture was used as treatments.

Among all tested explants sterilization treatments 2% *NaOCl* by double sterilization for 20 and 10 min and 70% ethanol for 10 min was 100% efficient for all tested varieties. The analysis result of data for shoot initiation showed that there was significance difference due to interaction effects of hormones and varieties for days to shoot initiation at ($p < 0.05$). For days of shoot initiation experiment, MS basal medium supplemented with all concentration levels of BAP alone or mixed with NAA took minimum days compared to control. However, the earliest shoot initiation was observed when MS medium supplemented with 2.5 mg/l BAP in combination with 0.5 mg/l NAA on Yanbule and for Mesena variety early shoot initiation also recorded at this treatment.

For shoot multiplication experiment, the analysis of the result indicated that there was a highly significant difference ($p<0.0001$) among the hormones, varieties as well as their interaction in terms of all shoot multiplication parameters except number of leaves per shoot. But the highest number of shoot per explant was observed on Yanbule at 5 mg/l BAP, for Mesena at 6.5 mg/l BAP and for Endale at 6.5 mg/l Kn alone. Whereas, maximum length of shoot per explant was observed at 2 mg/l BAP and 5 mg/l BAP for Yanbule and Mesena, respectively whereas the highest shoot length per explant for variety Endale was obtained at 3.5 mg/l Kn. As compared to other treatments the highest number of leaves per shoot was also observed on Yanbule at media enriched with 3.5 mg/l BAP. Similarly, significantly the higher shoot fresh and dry weight per shoot was recorded at 3.5 and 2 mg/l BAP respectively on Yanbule variety.

Different concentration of NAA and IBA alone or in combination with BAP positively enhanced the *in vitro* rooting of enset as compared to control. Analysis of Variance showed varieties and hormone interaction had highly significant ($p < 0.0001$) effect on Days of Root induction and Length of Root. But number of roots per shoot and Root Induction Percentage was influenced due to hormone and variety difference at ($P<0.05$). IBA at 3 mg/l in combination with 0.25 mg/l BAP on half strength MS was the most effective in roots number per shoot, root length per shoot and root induction percentage response of Yanbule than other treatments. However, number of roots per shoot was significantly

higher at 4.5 mg/l NAA for variety Endale. While, in Mesena significantly higher number of roots per shoot, root induction percentage and minimum days for root emergency recorded at 3 mg/l NAA. In case of acclimatization experiment plantlets acclimatized on red soil and sand mixture at 2:1 ratio was 100 % survived for all tested varieties.

In conclusion, this protocol can be utilized to micro-propagate the three enset varieties to boost provision of good quality planting material. In this study efficient explants sterilization, shoot initiation and multiplication, root induction and plantlets acclimatization protocols were optimized. The use of 2 mg/l BAP and 3.5 mg/l Kn resulted higher shoot multiplication as compared to other treatments for Yanbule and Mesena respectively. 1.5 mg/l and 3 mg/l NAA found to be best for root formation of Endale and Mesena respectively than IBA alone or in combination with BAP.

In general the current study revealed that there is a notable variation among genotypes of enset in performance during tissue culture propagation. Among the tested varieties Yanbule was found the best responsive followed by Mesena and Endale. Perhaps further refinement of the current protocol might be necessary to improve the *in vitro* propagation of the least responsive varieties under this study. These suggesting that, varietal difference evokes varying *in vitro* response to the same plant growth media.

5.2. Recommendations

The protocol raised in the present attempt could be used for the massive *in vitro* production of disease free plantlets of these elite enset varieties to disseminate for enset growers.

In the mean time, it is recommended that further study on the other factors such as media pH, other growth hormones, explants age and low cost technologies which replace sucrose as carbon source is necessary to improve the success to a higher level of propagation efficiently by narrowing the gap observed between the different genotypes.

The optimization study should also need to continue on the other elite genotype of enset to address the needs of all enset growers.

6. REFERENCES

Adane Gebeyehu. 2013. Effect of different combinations of 6-benzyl amino purine and naphthalene acetic acid on multiple shoot proliferation of plantain (*Musa* spp.) cv. Matoke from meristem derived explants. *African Journal of Biotechnology*, 12(7):709-719.

Admassu Tsegaye and Struik, P. C. 2001. Enset (*Ensete ventricosum (Welw.*) Cheesman) *kocho* yield under different crop establishment methods as compared to yields of other carbohydrate rich food crops. *Netherlands Journal of Agricultural Science,* 49(3): 81–94.

Admassu Tsegaye, Asnaketch Welde-tensaye, Brandt, A.S., Endale Tabogie, Gizachew Weld Michael, Kidist Bobosh, McCabe, T.J., Mulugeta Diro, Tesefaye Shiferaw , Shigeta Masayohi, Spring, A. and Yewelsew Abebe. 1997. Phase II Final Report of the Enset Needs Assessment Project. Awassa, Ethiopia.

Admassu Tsegaye. 2002. On indigenous production, genetic diversity and crop ecology of enset (*Enset ventricosum* (Welw.) Cheesman). PhD dissertation, Wageningen University.The Netherlands.

Afza, R., van Dure, M. and Morpurgo, R. 1996. Regeneration of *Ensete ventricosum* through somatic embryogenesis and adventitious buds. *Plant Cell Reports*, 15(4): 445-448.

Aish Muhammad, Hamid Rashid, and Iqbal Hussain. 2007. Proliferation-rate effects of BAP and Kinetin on Banana (Musa spp. AAA Group) ‘Basrai’. *Hortscience*, 42(5) : 1253–1255.

AL-amin, md. , Karim, m. R., Amin, M. R., Rahman, S. and Mamun, A. N. M. 2009. *In vitro* micropropagation of banana (*musa spp.*). *Bangladesh J. Agril. Res*., 34(4): 645-659.

Almaz Negash, Admassu Tsegaye, van Treuren, R. and Visser, B. 2002. AFLP Analysis o f Enset Clonal Diversity in South and South western Ethiopia for conservation. *Cr op Sci*., 42: 1105-1111.

Almaz Negash, Puite, K., Schaart, J., Visser, B. and Krens, F. 2000. *In vitro* regeneration and micropropagation of enset from Southwestern Ethiopia. *Plant Cell Tissue Org. Cult.*, 62: 153–158.

Amornwat Srangsam and Kamnoon Kanchanapoom. 2007. Establishment of *in vitro* Culture of Musa AA Group 'Kluai Sa' and Musa AA Group 'Kluai Leb Mue Nang' and the Analysis of Ploidy Stability. *Science Asia*, 33: 437-442.

Anbazhagan, M., Balachandran, B. and Arumugam, K. 2014. *In vitro* propagation of *Musa* sp (Banana). *International journal of current microbiology and applied sciences*, 3 (7): 399-404.

Archido, J.Q. 1991. First Quarter Report of the plant pathologist: July 1, to Sept 30, 1991. Enset team support project. Sidamo Gamo Goffa peasants Agricultural Development Program(PADEP)-III.Awassa research center(IAR), Awass Ethiopia.

Arias, O. 1992. Commercial micropropagation of banana. In: Biotechnology Applications for Banana and Plantain Improvement. *INIBAP, San Jose, Costa Rica*, pp. 139-142.

Arinaitwe, G., Rubaihayo, P.R., and Magambo, M.J.S. 2000. Proliferation rate effects of cytokinins on banana (Musa spp.) cultivars. *Scientia Hort*., 86:13–21.

Arnaldos, T., Munoz, L. R., Ferrer, M.A. and Calderon, A.A. 2001. Changes in phenol content during strawberry (*Fragaria* x *ananasa*, cv. Chandler) callus culture. *Physiol. Plant.,*113(2): 315–322.

Asnakech Welde-Tensaye. 1997. The Ecology and Production of *Ensete ventricosum* in Ethiopia.Doctoral thesis, Swedish University of Agriculture Science. *Uppsala.* PP. 129 .

Atlabachew Minaleshwa and Chandravanchi, B. S. 2008. Levels of major, minor and trace elements in commercially available enset (*Ensete ventricosum* (Welw.) Cheesman) food products (*Kocho and Bulla*) in Ethiopia. *Journal of Food Composition and Analysis,* 21: 545–552.

Baker FS, Miller CE, Repik AJ and Tolles ED. 1992. Activated carbon. *Kirk-Othmer Encycl Chem Technol*, 4: 1015–1037.

Baker, R. E. D. and Simmonds, N. W. 1953. The genus *Ensete* in Africa. *Kew Bulletin,* 8: 405–416.

Bezuneh Taye. 2010. Technological challenges of sustainable enset farming system: enhancing production of food/fiber and industrial outputs. In: Enset Research and Development Experiences in Ethiopia 2010. Ethiopian Institute of Agricultural Research (EIAR), Wolkite: p.1-20.

Bezuneh Taye. 1996. An overview on enset research and future technological needs for enhancing its production and utilisation. *In:* Abate Tsedeke, Hiebsch C, Brandt S.A. and Gebremariam Seifu (eds), Enset based Sustainable Agriculture in

Ethiopia: Proceedings from the International Workshop on Enset, pp: 1-14.13-20 December 1993, Institute of Agricultural Research, Addis Ababa.

Bhojwani, S.S. and Razdan, M.K. 1996. *Plant Tissue Culture: Theory and Practice*. Amsterdam, Netherland, Elsevier Science B.V, pp.767.

Bizuayehu Tesfaye. 2002. Studies on Landrace diversity, *in vivo* and *in vitro* Regeneration of Enset (*Enset ventricosum* Welw.). PhD dissertation, Humboldt University, Berlin, Germany.

Bizuayehu Tesfaye. 2008. The enset (*Ensete ventricosum*) gardens of Sidama: compositio n, structure and dynamics of a traditional poly-variety system. *Genetic Resources and Crop Evolution,* 55: 1347–1358.

Brandt, S. A., Spring, A., Hiebsch, C., McCabe, J. T., Endale Tabogie, Muligeta Diro, Gizachew Wolde Michael ,Yntiso Gebre, Shiegeta Masayoshi and Tesfaye Shiferaw.1997. The "Tree Against Hunger": Enset- Based Agricultural Systems in Ethiopia. American Association for the Advancement of Science, Washington.

Brugiere, N., Jiao S., Hantke, S. Zinselmeier, C., Roessler, J.A. Niu, X. Jones, R.J. and Habben, J.E. 2003. Cytokinin oxidase gene expression in maize is localized to the vasculature, and is induced by cytokinins, abscisic acid, and abiotic stress. *Plant Physiology,* 132: 1228-1240.

Buah JN, Danso, E., Taah KJ, Abole EA, Bediako EA, Asiedu, J. and Baidoo, R. 2010. The effects of different concentration cytokinins on the *in vitro* multiplication of plantain (Musa sp.). *Biotechnology*, 9(3): 343- 347.

Buising C. M., Shoemaker R. C. and Benbow R. M. 1994. "Early Events of Multiple Bud Formation and Shoot De-velopment in Soybean Embryonic Axes Treated with the Cytokinin, 6-Benzylaminopurine," *American Journal of Botany*, 81(1): 1435-1448.

Cardoza, V. 2008. Tissue Culture: The Manipulation of Plant Development. pp. 113-133 *In*: C.N. Stewart (Ed.) *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*. Hoboken, New Jersey John, USA, Wiley & Sons, Inc.

Central Potato Research Institute (CPRI). 1992. Tissue Culture technique for potato health, conservation, micro propagation and improvement. *CPRI*: 1-23.

Central Statistical Agency (CSA). 2014. Area and Production of Major Crops. The federal democratic republic of Ethiopia; Agricultural Sample Survey report.CSA, vol.1. Addis abeba, Ethiopia.

Centeral Statistical Agency (CSA). 2016. Agricultural Sample Survay. 2015/2016: cropping season report on Area and Production of Major Crops. VI, *Statistical Bulletin* 584; Addis abeba, Ethiopia.

Chakrabarty, D., Chatterjee, J. and Datta, S.K. 2007. Oxidative stress and antioxidant activity as the basis of senescence in chrysanthemum florets. *Plant Growth Regul*, 53: 107-115.

Cheng zj, zhu ss, gao xq, and zhang xs. 2010. Cytokinin and auxin regulates WUS induction and inflorescence regeneration *in vitro* in *arabidopsis. Plant cell reports*, 29: 927–933.

Cronauer, S.S. and Krikorian, A.D. 1984. Multiplication of Musa from excised stem tips. *Annals of Botany*, 53 (3): 321-328.

Dagmawit Tobiaw and Endashaw Bekele . 2011. Analysis of genetic diversity among cultivated enset (*Ensete ventricosum)* populations from Essera and Kefficho, south western part of Ethiopia using inter simple sequence repeats (ISSRs) marker. *Afr. J. Biotechnol*., 10(70): 15697-15709.

Dayarani,M., Dhanarajan, M.S, and Uma, S. 2013. *In-Vitro* Response of Ornamental Banana (Musa Spp.). *International Journal of Chemical, Environmental and Biological Sciences (IJCEBS)*, 1(3): 2320-4079.

Dunstan, D.I. and Turner, K.E. 1984. The acclimatization of micropropagated plants. In cell culture and Somatic cell Genetics of plants. Vol. I Laboratory procedures and their applications. *Vasil, I.K. (Ed), Academic Press, Oriando.* 123-129.

Edwin F. George, Michael A. Hall and Geert- Jan De Klert. 2008. Plant Propagation by Tissue Culture, 3rd Edition. Springer Published, The Netherlands.

Engelmann, F. 2010. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular and Developmental Biology Plant,* 47 (1): 5-16.

Ethiopian Science and Technology Commission (ESTC). 2003. Commission awards individuals for outstanding achievements. http://www.capitalethiopia.com/archive */2003/july/week3/index.htm.*

Ethiopian Institute of Agricultural Research (EIAR). 2013. http://www. *eiar.gov.et/research centre/3 federal research center/26 holetta agricultural research centre*, Accessed on 25 Octo 2015.

Feng, J.T., Zhi-yi, Z., Jun, z., Na, Y. and Dmei, W. 2007. Contamination and Browning in tissue culture of (Platanus Occidentalis L.). *For. Stud. China*, 9(4): 279-282.

Ferdous, M. H., Masum Billah, A. A., Mejraj, H., Taufique, T. and Jamal Uddin, A. F. M. 2015. BAP and IBA pulsing for *in vitro* multiplication of banana cultivars through shoot-tip culture. *Journal of Bioscience and Agriculture Research,* 3(2): 87-95.

Garg, P. 2011. Plant tissue culture of *Jatropha curcasl*: A review. *International Imperial Journal of Pharmacognosy and Natural Products* 1 (1): 6-13.

Gaspar, T., Kevers, C. Penel, C. Greppin, H. Reid, D.M. and Thorpe, T.A. 1996. Plant hormones and plant growth regulators in plant tissue culture: Review. *In Vitro Cellular and Developmental Biology Plant,* 32: 272-280.

Genene Gezahegne. 2014. *In vitro* regeneration of disease free enset [*ensete ventricosum* (welw.) cheesman] planting materials from bacterial wilt diseased plants using shoot tip culture. M.S.C Thesis Haremaya University, Ethiopia.

Genet Birmeta and Welander, M. 2004. Efficient micro propagation of *Ensete ventricosum* applying meristem wounding: a three step protocol. *Plant Cell Reports*, 23(5): 277-283.

Genet Birmeta, Nybom H, and Endashaw Bekele. 2004. Distinction between wild and cultivated enset *(Ensete ventricosum*) gene pools in Ethiopia using RAPD markers. *Hereditas*, 140: 139-148.

George, E.F., Hall, M.A. and Klerk, G.J.D. 2008. *Plant Propagation by Tissue Culture 3rd Edition*. Dordrecht, Netherlands, Published by Springer, pp. 501.

George, E.F. 1993. Plant Propagation by Tissue Culture. Part 1 the Technology. 2nd ed, Ex egetics Ltd. England, p294.

Gonzales, R.A. 1994. Plant Cell Culture: A Practical Approach. Oxford University Press, Oxford.

Gubbuk, H. and Pekmezci, M. 2004. *In Vitro* Propagation of Some New Banana Types (Musa spp.). *Turkish Journal of Agriculture and Forestry*, 28: 355-361.

Holscher, D. and Schneider, B. 1998. Phenylphenalenone from *Ensete ventricosum. Phyto chemistry Oxford,* 49: 2155-2157.

Hopkins, W.G. and Hüner, P.A. 2009. *Introduction to Plant Physiology Fourth Edition*. 111 River Street, Hoboken, London, John Wiley and Sons, Inc., pp. 503.

Ishtiaq Ahmad, Tanveer Hussain, Irfan Ashraf, Muhammad Nafees, Marym rafay and Muhammed Iqbal. 2013. Lethal Effects of Secondary Metabolites on Plant tissue culture. *American Eurasian J. Aric and Environ. Sci.*, 13(4): 539-547.

Karlsson, L. M., Tamado Tena, Dalbato, A. L. and Mikias Yeshitila. 2013. Seed morphology and germination of *Ensete ventricosum* (Musaceae). *Seed Science and Technology*, 41: 357–370.

Khalafalla,M.M., Abdellatef, E., Mohamed Ahmed, M.M. and Osman, M.G., 2007. Micropropagation of sweet potato (*I.batatas*) grows regularly. *International Journal for sustainable Crop Production*, 2: 1-8.

Khalid, N. 2011. Effect of Benzylaminopurine (BAP) pulsing on *in Vitro* Shoot Multiplication of *Musa acuminate* (Banana) cv. Berangan. *African Journal of Biotechnology,* 10: 2446-2450.

Khanna, H.K. and Raina, S.K. 1998. Genotype x culture media interaction effects on regeneration response of three *indica* rice cultivars. *Plant Cell, Tissue and Organ Culture*, 52(3): 145-153.

Kumar, K. and Rao, I.U. 2012. Morphophysiologicals problems in acclimatization of micropropagated plants in-*ex vitro* conditions- A Reviews. *Journal of Ornamental and Horticultural Plants* 2 (4): 271-283.

Lalremsiami Hrahsel and Robert Thangjam. 2013. Strategies for large scale production of commercially important banana varieties of Mizoram, India, using plant tissue culture technique. *Sci.Vision*, 13 (3); Research Forum in India.

Leng P, Su S, Wei F, Yu F and Duan Y. 2009. Correlation between browning, total phenolic content, polyphenol oxidase and several antioxidation enzymes during pistachio tissue culture. *Acta Hort*., 829: 127-132.

Madhulatha, P., Anbalagan, M., Jayachandran, S. and Sakthivel, N. 2004. Influence of Liquid Pulse Treatment with Growth Regulators on *in Vitro* Propagation of Banana (*Musa* spp. AAA). *Plant Cell*, *Tissue and Organ Culture*, 76(5): 189-192.

Mathew MM and Philip VJ. 1996. Clonal propagation of Enset (*Ensete superbum* (Roxb.). Cheesman) through shoot tip cultures. *Plant Cell Report*, 16: 232–234.

Mathur, S. 2013. Conservation of biodiversity through tissue culture. Research and Reviews: *Journal of Microbiology and Biotechnology,* 2 (3): 1-6.

Mendes B. M. J., Mendes F. J., Tulmann Neto A., Demétrio CGB and Pieske OR. 1996. Efficacy of banana plantlet production by micro propagation. *Pesqui Agropecu Bras,* 31: 863–867.

Mikias Yeshitila. 2014. Cluster analysis for evaluation of genetic diversity in Enset (*Enset ventricosum* (Welw.) Cheesman) clones at Areka Condition. *J. Plant Sci*., 2(1): 55-69.

Ministry of Agriculture (MOA). 2010. Crop variety register. Animal and Plant health Regulatory directorate, Issue no 13, Addis Ababa, Ethiopia.Pp 154.

Ministry of Agriculture and Rural Development (MOARD). 2009. Crop variety register. Animal and Plant health Regulatory directorate: Issue no 12, Addis Ababa, Ethiopia. Pp. 127.

Mohammed, B., Gabel, M. and Karlsson, L. M. 2013. Nutritive values of the drought tolerant food and fodder crop enset. *African Journal of Agricultural Research,* 8: 2326–2333.

Molla, M.H., Khanam, M., Amin, M. Al. and Malek, M.A. 2004. In vitro rooting and ex vitro plantlet establishment of BARI Banana 1 (Musa sp.) as influenced by different concentration of IBA (Indole 3-butyric Acid). *Asian Journal of Plant Sciences,* 3(2):196-199.

Morpurgo R, Afza R and Novak FJ. 1996. Biotechnology and enset achievements and perspectives. *In:* Abate T, Hiebsch C, Brandt SA and Gebremariam S (eds), Proceedings from the International Workshop on Enset, pp 256-270. 13-20 December 1993, Addis Ababa

Muhammad Aish, Hussain Iqbal, Naqvi S.M. and Rashid Hamid. 2004. Banana Plantlet Production through Tissue Culture. *Pak. J. Bot*., 36(3): 617-620.

Muhammad, M.I, Aish, M., Iqbal, H., and Hazrat, B. 2013. Optimization of *in vitro* micropropagation protocol for banana (*Mussa sapientum L.)* under different hormonal concentrations and growth media. *IJAIR,* 2(1):2319-1473.

Mulugeta Diro , Gebremariam Seifu, Zelleke Adugna and Van Staden, J. 2002. Growth of enset (*Ensete ventricosum*) suckers under different horticultural practices. *South African Journal of Botany,* 68: 430-433.

Mulugeta Diro and Van Staden, J., 2004. The type of explants plays a determining role in the micro propagation of Ensete Ventricosium. *South African Journal of Botany, 71(2): 154-159.*

Mulugeta Diro and Van staden, J. 2003. *In vitro* regeneration of *Ensete ventricosum* from z ygotic embryos of stored seed. *South African journal of Botany*, 69: 364-369.

Munguatosha, N., Emerald, M. and Patrick, N. 2013. The Effects of Auxins and Cytokinin on Growth and Development of (Musa sp.) Var. “Yangambi” Explants in Tissue Culture. *American Journal of Plant Sciences*, 4: 2174-2180.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 219-223.

Negash Mesele, Yirdaw Eshtu and Luukkanen, Olavi. 2012. Potential of indigenous multistrata agroforests for maintaining native floristic diversity in the south-eastern Rift Valley escarpment, Ethiopia. *Agroforestry Systems*, 85: 9–28.

Nguyen TV, Thu, T.T., Claeys, M. and Angenon, G. 2007. Agrobacterium mediated transformation of sorghum (*Sorghum bicolour (L.)* Moench) using an improved *in vitro* regeneration system. *Plant Cell Tissue Organ Cult.,* 91: 155–64.

North, J., Ndakidemi, P. and Laubscher, C. 2012. Effects of Antioxidants, Plant Growth Regulators and Wounding on Phenolic Compound Excretion during Micro propagation of Strelitzia Reginae. *International Journal of Physical Sciences*, 7(4): 638-646.

Oyebanji, O.B., Nweke, O., Odebunmi, O., Galadima, N.B., Idris, M.S., Nnodi, U.N., Afolabi, A.S. and Ogbadu, G.H. 2009. Simple, effective and economical explants surface sterilization protocol for cowpea, rice and sorghum seeds. *African Journal of Biotechnology*, 8(20): 5395-5399.

Ozyigit, I.I. 2008. Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum L*) shoot tips. *Afri. J. Biotechnol.*, 7: 1145 – 1150.

Pasqual, M., Soares, J.D. and Rodrigues, F.A. 2014. Tissue culture applications for the genetic improvement of plants, pp. 157-199. *In*: A. Borém and R. Fritsche-Neto (Ed.)

Pierik, Rlm. 1993. Micropropagation: Technology and opportunities. In: Prakash J and Pierik RLM (eds), Plant Biotechnology: *Commercial Prospects and Problems*, pp 8-15. Intercept Ltd, UK.

Pursglove, J.W. 1972. Tropical Crops, Monocotyledons. Holsted Press, *A division of John Wiley and Sons, Inc. New York*. pp. 243-244.

Quimio, A.J. and Mesfine Tessera. 1996. Diseases of enset. In: Tsedeke Abate., Hiebsch, C., Brandt, S.A., Gebremariam Seifu (Eds.), pp. 188-203. Enset Based Sustainable Agriculture in Ethiopia. *Proceedings of the International Work shop on enset.* Addis Ababa, Ethiopia, 13-20 December 1993.

Rahman, M.Z., Rahaman, M.H., Mullah, M.U., Nahar, N., Sultana, R.S., Bari, M.A. and Hossain, M. 2005. In vitro shoot multiplication and rooting of a dessert banana(Musa sp cv. Anupom). *Pakistan journal of biological science*,8(9): 1298-1302.

Rai, R. 2007. *Genetics and Plant Breeding: Introduction to Plant Biotechnology*. National Research Centre on Plant Biotechnology, Lal Bhadur Shastri Building Pusa Campus, New Delhi, India.

RAO, P.S. 1994. Biotechnological approaches for increasing plant productivity. *AsiaTech.* , 3: 32-36.

Resmi, L. and Nair AS. 2007. Plantlet production from the male inflorescence tips of Musa acuminata cultivars from South India. *Plant Cell Tissue Organ Cult*., 88: 333-338.

Reza, F., Kamaludin, R., Rosna MT and Jamilah SY. 2013. Effective Sterilization Protocol for Micropropagation of *Musa coccinea* (*Musa SPP*). *International Conference on Agriculture and Biotechnology. vol.60.* Singapore.

Rout, G. R. T., Samantaray, S. and Das, P. 2000. "*In Vitro* Ma-nipulation and Propagation of Medicinal Plants". *Bio-technology Advances*, 18(2): 91-120.

Shahnawaz Ahmed, Akash Sharma, Singh, A.K., Wali, V.k. and Preeti Kumar. 2014. *In vitro* multiplication of banana (Musa sp.) cv. Grand Naine. *Plant Tissue Cult*., 13(27): 2696-2703.

Shank, R. and Ertiro, C. 1996. A linear model for predicting Enset plant yield and assessment of *Qocho* production in Ethiopia. World Food Programme/SNNPRS units for Ethiopia. *Http://www.africa.upenn.edu/eue_web/Enset.htm.*

Shank, R. 1994. The Enset Culture, Technical Report on Enset or the False Banana.United Nations-Emergencies Unit for Ethiopia.

Short, KC. 1990. Application of *in vitro* techniques for the production and the improvement of horticultural plants. In: Sangwan Sangwan Norreel, Current Plant Science Biotechnology: The Impact of Biotechnology in Agriculture. Proceedings of the International Conference 'The Meeting Point between Fundamental and Applied *In Vitro* Culture Research'. *Kluwer Academic Publishers*, 5: 15-27.

Sim, S.T. 2006. Virus elimination from grape selections using tissue culture. *Foundation Plant Services Grape Program Newsletter*.

Simmonds, N. W. 1962. The evolution of the bananas (2nd Ed.). Longmans, London.

Simmons, F.J. 1956. The role of Ensete in Ethiopia. *The Geographical Review*, 46:271-272.

Soniya EV and Sujitha, M. 2006. An efficient *in vitro* propagation of *Aristolochia indica*. *Biol Plant*, 50: 272–274.

Southern Agricultural Research Institute (SARI). 2014. "Integrated Management of Bacterial Wilt of Enset (*Ensete ventricosum* (Welw.) Cheesman) caused by

Xanthomonas campestris pv. *musacearum* in Ethiopia". The McKnight Foundation Collaborative Crop Research Project, Annual report (November 2013 to October 2014); Ethiopia.

Spring, A. 1996. Enset farming system in Southern Region, Ethiopia. Report on a rapid rur al appraisal in Gurage, Hadiya and Sidama Zones. Enset Need Assessment Project Phase I, Awasa, Ethiopia.

Srivastava, N., Kamal, B., Sharma, V., Negi, Y.K., Dobriyal, A.K., Gupta, S. and Jadon, V.S. 2010. Standardization of Sterilization Protocol for Micro propagation of *Acon itum heterophyllum.*An Endangered Medicinal Herb. *Academia Arena,* 2(6): 37-40.

Taye Bezunehe.1984. Evaluation of some *Ensete ventricosum* clones for food yield with emphasis on the effect of length of fermentation on carbohydrate and calcium content. *Tropical Agriculture*, 61(2): 111-116.

Temesegen Magule , Bizuayehu Tesfaye, Marcello Catellani and Mario Enrico Pe. 2014. Indigenous knowledge,use and on-farm management of enset (*Ensete ventricosum* (Welw.) Cheesman) diversity in Wolaita, Southern Ethiopia. *J. Ethnobiol Ethnomed.*, 10: 1-18.

Temesgen Magule , Bizuayehu Tesfaye, Mario Augusto Pagnotta, Mario Enrico Pe and Marcello Catellani. 2015. Development of SSR markers and genetic diversity analysis in enset (*Ensete ventricosum* (Welw.) Cheesman), an orphan food security crop from Southern Ethiopia. *BMC Genetics,* 16: 98. *doi: 10.1186/s12863-015-0250-8.*

Thomas, T.D. 2008. The Role of Activated Charcoal in Plant Tissue Culture. Research review paper; *Biotechnology Advances*, 26: 618-631.

Tsehaye Yemane and Kebebew Fassil. 2006. Diversity and cultural use of enset (*Ensete ventricosum (Welw.)* Cheesman) in Bonga in situ conservation site, Ethiopia. *Ethnobotany Research and Applications*, 4: 147–157.

Tyburski, J., and Tretyn, A. 2004. The role of light and polar auxin transport in root regeneration from hypocotyls of tomato seedling cuttings. *Plant Growth Regulation,* 42: 39–48.

Vanisree, M., Lee, C. Lo, S. Nalawade, S.M. Lin, C.Y. and Tsay, H. 2004. Studies on the production of some important secondary metabolites from medicinal plants by planttissue cultures. *Botanical Bulletin of Academia Sinica* 45: 1-22.

Vavilov NI. 1951. The origin, variation, immunity, and breeding of cultivated plants. *Chiron Bot.,* 13: 1-366.

Vuylsteke, D.R. 1989. Shoot-tip culture for the propagation, conservation and exchange of *Musa* germplasm, International Board for Plant Genetic Resources, Rome.

Westphal, E. 1975. Agricultural system in Ethiopia.Centre for Agricultural Publishing and Documentation, Agricultural University, Wageningen.The Netherlands.

Woeste, K.E., Vogel, J.P. and Kieber, J.J. 1999. Factors regulating ethylene biosynthesis in etiolated Arabidopsis thaliana seedlings. *Physiologia Plantarum* 105: 478-484.

Zeweldu Tilahun. 1997. Comparative Tissue Culture Study on Banana and Plantain *(Musa* spp.) and Development of *In Vitro* Methods for Propagation of *Enset (Ensete* spp.). PhD Thesis, Humbolt University of Berlin, Verlag Dr.Koster, Berlin.

Zeweldu Tilahun and Ludders, P. 1998. Preliminary tissue culture investigation in *Ensete* (Ensete spp.). *Journal of Applied Botany*, 72: 25-27.

7. APPENDICES

Appendix A: List of components in MS medium and plant growth regulators

Appendix Table 1. Nutrient composition and concentration of full strength MS medium

MS media component	Concentration (g)	Amount of stock solution taken for one litre medium (ml)
MS 1	**For 1000 ml**	
Ammonium Nitrate (NH_4NO_3)	33	**50**
Potasium Nitrate (KNO_3)	38	
MS2	**For 500ml**	
Magnesium Sulphate($MgSO_4.7H_2O$)	18.07	**5**
Manganese Sulphate ($MnSO_4.H_2O$)	1.69	
Zinc Sulphate ($ZnSO_4.7H_2O$)	0.86	
Copper Sulphate ($CuSO_4.5H_2O$)	0.0025	
MS 3	**For 500 ml**	
Calcium Chloride ($CaCl_2.2H_2O$)	33.22	**5**
Potassium Iodide (KI)	0.083	
Cobalt Chloride ($CoCl.6H_2O$)	0.0025	
MS4	**For 500 ml**	
Potassium dibasic Phosphate (KH_2PO_4)	17	**5**
Boric Acid (H_3BO_3)	0.62	
Sodium Molbdate ($Na_2MoO_4.2H_2O$)	0.025	
MS5	**For 500 ml**	
Na2EDTA	3.72	**5**
Iron Sulphate ($FeSO_4.7H_2O$)	2.78	
MS6	**For 500 ml**	
Myoinositol	10	**5**
Glycine	0.2	
Thiamine HC	0.01	
Pyridoxine HCl	0.05	
Nicotinic acid	0.05	

Appendix Table 1. Continud

Other Organic Supplements(gm/l) for media	
Sucrose	30
Activated Charcoal	1
Agar Agar type	4.5 or 5
Cefataxime	0.5
Ascorbic acids	0.025

Appendix Table 2. Plant growth regulators hormone supplied in the media

Plant growth regulators	Concentration(mg/ml) for stoke solution	Amount dispensed for 1 liter media(mg/l)
BAP	25	0.25, 1.5, 2, 2.5, 3, 3.5,4.5, 5, and 6.5
NAA	25	0.5, 1, 1.5, 3 and 4.5
IBA	25	1.5, 3 and 4.5
Kn	25	2, 3.5, 5 and 6.5

Solvent: 95% ethanol and 1N NaOH

Appendix B: ANOVA Tables of explants sterilization, shoot initiation and multiplication and rooting experiment

Appendix Table 3. ANOVA Table for the effect of *NaOCl* on clean and live explants culture

Source of variation	DF	Mean Square
Varieties	2	54.11^{ns}
NaOCl	3	17547.7***
Varieties* *NaOCl*	6	285.49*
Error	23	108.69
CV		18.24

*= Significant, ***=highly significant, ns= none significant, CV=Coefficient of variation, DF= degree of freedom

Appendix Table 4. ANOVA Table for Shoot Initiation

Source of variation	DF	Mean Square
		Date of Shoot Initiation
Varieties	2	39.24***
Hormones	12	14.53***
Varieties*Hormones	24	0.99*
Error	77	1.15
CV		10.7

*= Significant, ***=highly significant, CV=Coefficient of variation, DF= degree of freedom

Appendix Table 5. ANOVA Table for *in vitro* Shoot multiplication

Source of variation	DF	Mean Square				
		Number of Shoots/explant	Length of Shoot(cm)	Number of Leaves/shoot	Shoot Fresh Weight /Shoot(g)	Shoot Dry Weight/Shoot(g)
Varieties	2	17.64***	111.9***	15.89***	14.02***	0.0391***
Hormones	12	11.74***	4.84***	0.62***	1.02***	0.0021***
Var*Horm	24	4.41***	2.05***	0.17**	0.31***	0.0007***
Error	77	0.134	0.161	0.073	0.021	0.000073
CV		12.9	10.2	11.6	14.4	15

=Significant, *= highly significant, CV= Coefficient of Variation, DF=degree of freedom

Appendix Table 6. ANOVA Table of rooting experiment

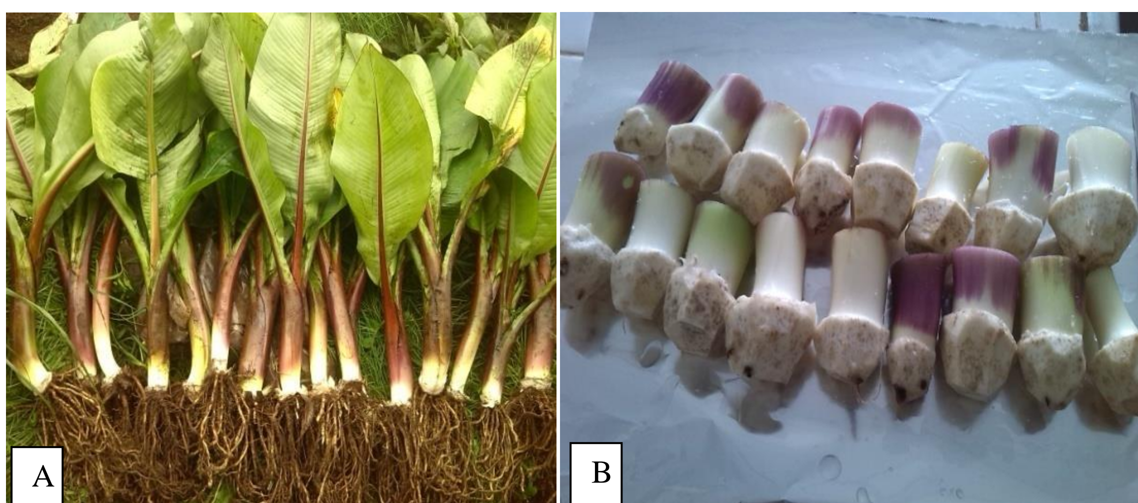
Source of variation	DF	Mean Square			
		Date of Root initiation	Number of Root/shoot	Length of Root/shoot(cm)	Root Induction Percentage (%)
Varieties	2	8.82***	0.39**	71.77***	3255.48***
Hormones	9	23.57***	2.07***	18.06***	373.73**
Var*Horm	18	5.08***	0.84***	1.95***	310.95***
Error	59	0.41	0.017	0.28	85.88
CV		5.9	12	10.5	19

=Significant, *= highly significant, CV= Coefficient of Variation, DF=degree of freedom

Appendix C: Photo gallery of mother plant establishment, explants sterilization, shoot initiation and multiplication, rooting and plantlets acclimatization experiments

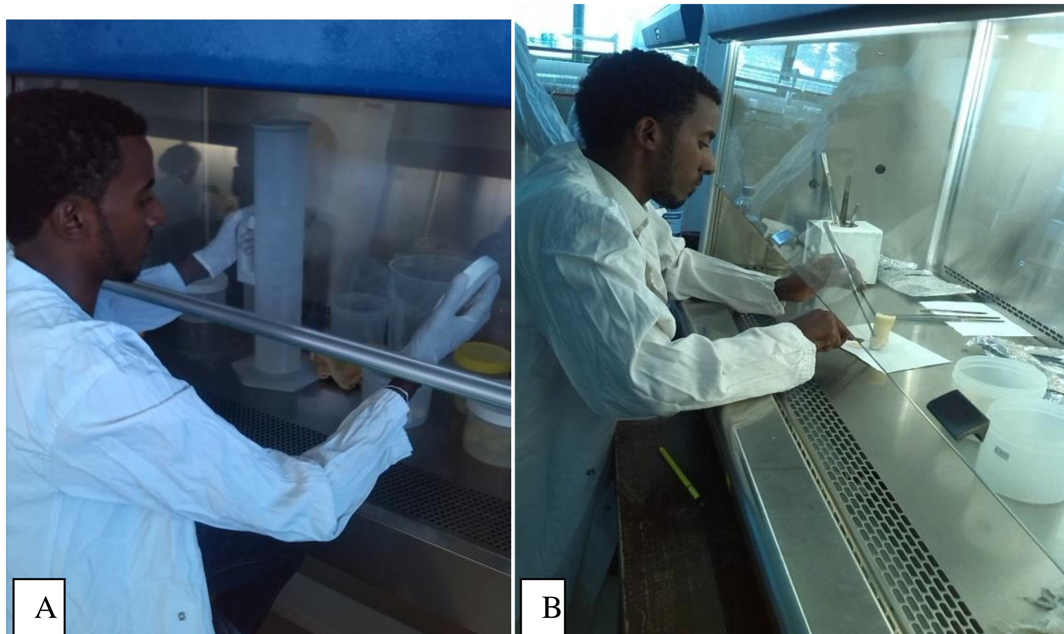
Appendix Figure 1. Mother plant decapitation, decortication and Suckers multiplication

Note; (A) Decapitated and Decorticated mother plant, (B) Multiplied suckers from mother plant



Appendix Figure 2. Five months old explant source stock plant and well prepard explant before sterilization

Note; (A) Stock plants, (B) Shoot with subtended corm

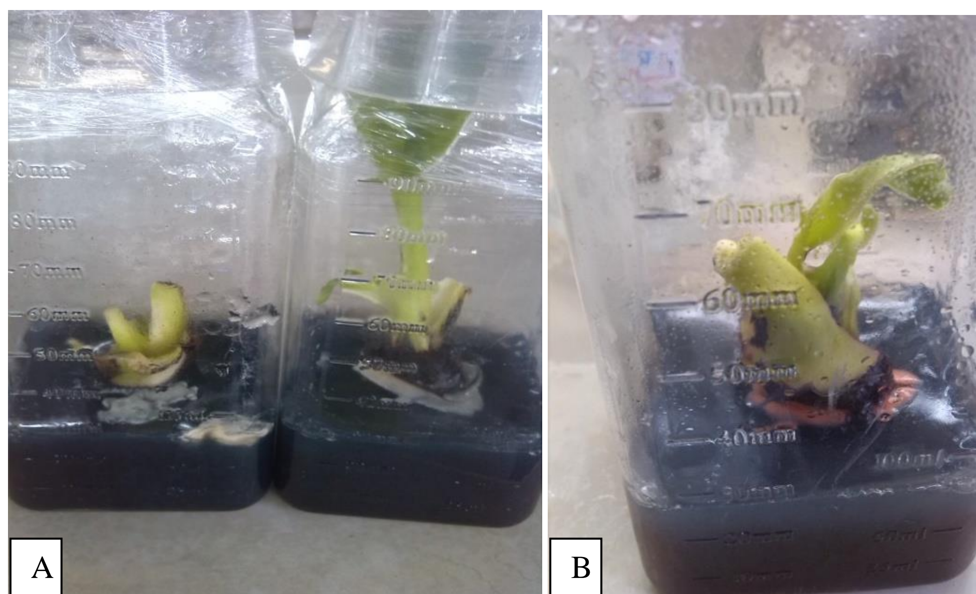


Appendix Figure 3. Aseptics sterilization techniques and explant trimming

Note, (A) Explants Sterilization with *NaOCl*; (B) Explant size reduction

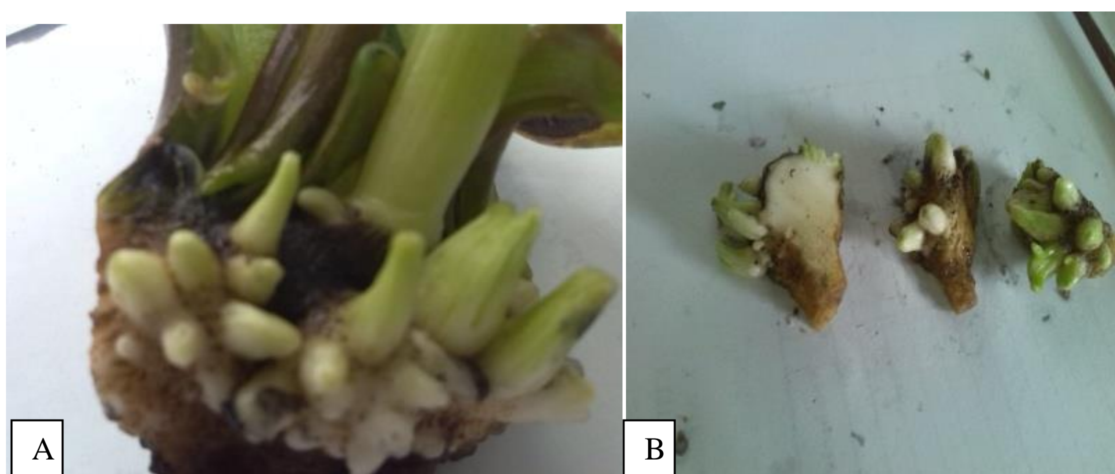
Appendix Figure 4. Inoculated shoot tip explant and Contamination free initiated culture explants at 2% *NaOCl*

Note; (A) Final size of inoculated explant, (B) Initiated culture



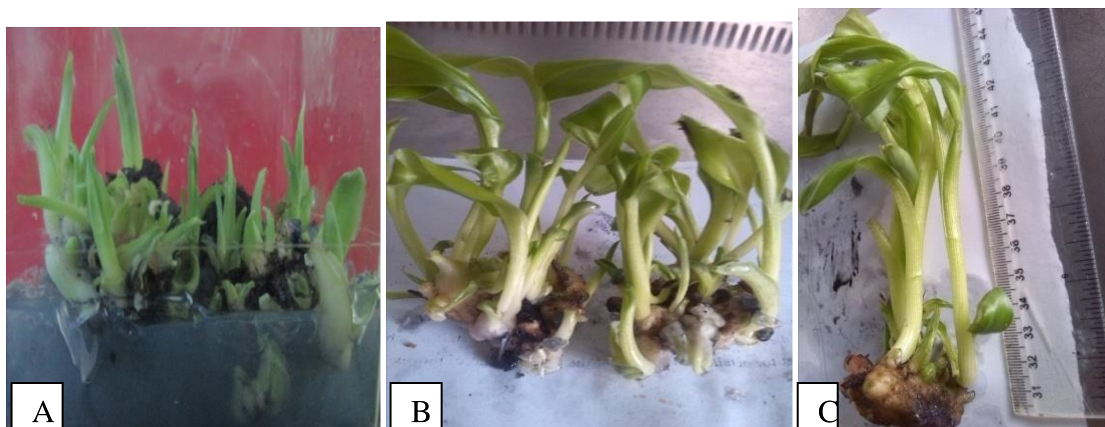
Appendix Figure 5. Contaminated shoot tip culture

Note; (A) Culture sterilized on *NaOCl* free, (B) Culture sterilized on 1% *NaOCl*



Appendix Figure 6. Active initiated bud and shoot after a month shoot initiation

Note;(A) Initiated bud and shoot from initial shoot tip explant, (B) Subculturing of initiated bud and shoot before shoot multiplication experiment

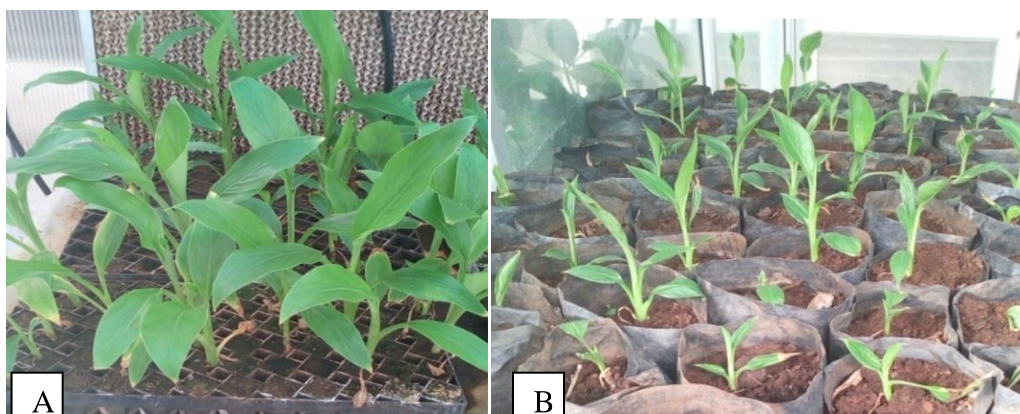


Appendix Figure 7. Multiplied shoots and Shoot length

Note;(A) Mesena at 6.5 mg/l BAP, (B) Yanbule at 5 mg/l BAP, (C) Yanbule shoot length at 2 mg/l BAP

Appendix Figure 8. Well rooted plantlets and root length

Note;(A) Yanbul at 3 mg/l IBA+0.25 mg/l BAP,(B) Mesena at 3mg/l NAA,(C) Endale root length 1.5 mg/l NAA



Appendix Figure 9. Acclimatized Plantlets in the green house

Note; (A) Aclimatized plantlets on Sand and red soil mixture, (B) Enset seedling in the polyethylene bag after acclimatization